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Magnetite magnetosome biomineralization in Magnetospirillum magneticum strain AMB-1: A time course study

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oxide speciation, from a more Fe(III)-rich species, possibly α -hematite (Fe_2O_3), to magnetite (Fe_3O_4), was observed in the early stages, with evidence for the presence of the Fe(III)-rich character persisting up to 24 h. The spectromicroscopy (X-ray absorption, XAS and X-ray magnetic circular dichroism, XMCD) and TEM results show that biomineralization occurs in a stepwise fashion. First, very small particles, with no measurable magnetization, are produced at different sites in the cell without significant chain formation. The Fe L_3 spectra of these early stage particles typically differ from magnetite with an additional signal at 708.4 eV that is consistent with α -hematite. By 6-8 h the particles are more numerous, partial chain formation is evident, and the L_3 spectrum is very similar to that of magnetite. By 24 h particles-in-chains are the dominant motif and magnetism with the moment along the chain is established. By 48 h the cells are essentially the same as cells grown in Fe-rich medium.

Keywords: magnetotactic bacteria; *Magnetospirillum magneticum* strain AMB-1; biomineralization; time course; transmission electron microscopy; synchrotron spectro-microscopy; STXM; X-ray absorption; X-ray magnetic circular dichroism

Highlights:

- * TEM and STXM study of magnetosome biomineralization in AMB-1 magnetotactic bacteria
- * time course method: cells cultured without Fe; placed in normal medium; sampled at 0.15, 0.5, 1, 2, 4, 5.7, 8, 12, 24, 28 h
- * TEM particle counts/cell; STXM measure Fe L X-ray absorption and X-ray magnetic circular dichroism
- * Fe(III) rich precursor, possibly α -hematite, observed in <8 h samples
- * pure magnetite particles aligned in chains form by 12 hr; gaps and magnetic reversals persist at 48 h

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5 70 **1. Introduction**
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7 71 Magnetotactic bacteria (MTB) biomineralize intracellular, single-magnetic-domain,
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9 72 single-crystal, membrane- bounded magnetite (Fe_3O_4) or greigite (Fe_3S_4) nanoparticles known as
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11 73 magnetosomes (Bazylinski and Frankel 1995). Magnetosomes are generally organized as a chain
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13 74 or chains within the cell (Bazylinski 1999). In this arrangement, they function as a strong
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15 75 magnetic dipole, so the cell passively aligns along the Earth's geomagnetic field lines like a
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17 76 compass needle. Coupled with aerotaxis (chemotaxis to the concentration of O_2),
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19 77 magneto-aerotaxis is believed to increase the efficiency of MTB in locating and maintaining
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21 78 their position at their optimal O_2 concentration and redox poise for survival in sediments or
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23 79 stratified water columns (Bazylinski 1999). The optimal O_2 concentration/redox conditions for
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25 80 most MTB appears to be at, or close to, the oxic-anoxic interface (OAI) (Faivre & Schuler 2008).
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27 81 MTB, which likely evolved in the mid-Archaeon, are of tremendous interest as possibly one of
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29 82 the earliest organisms on Earth capable of magnetic sensing and biomineralization (Lin *et al.*
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31 83 2017). They are a superb 'laboratory' for dissecting controlled biomineralization. They
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33 84 significantly impact the magnetic properties of sediments (Chen *et al.* 2015), and are implicated
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35 85 in the geochemical cycles of phosphorus (Rivas *et al.* 2018, Schulz-Vogt *et al.* 2019) and iron
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37 86 (Lin et al 2013, Lin et al 2014, Chen *et al.* 2014, Amor et al 2019). Through measurements of the
38
39 87 iron content of single AMB-1 cells, Amor et al (2019) showed that MTB absorb a significant
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41 88 fraction of the iron dissolved in their environment and thus play an important role in global iron
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43 89 distributions. Cells of a mutant lacking core genes for biomineralization also contained a
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45 90 significant (but decreased) amount of iron, indicating that magnetosomes are only one reservoir
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47 91 of iron among others in MTB.
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51 92 The genetic control of magnetosome biomineralization is an active area of study. A
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53 93 conserved core of essential genes has been identified (Komeili *et al.* 2004, Jogler and Schuler,
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55 94 2007, Arakaki *et al.* 2008, Murat *et al.* 2012). However, the chemical steps and reactions in
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57 95 magnetosome biomineralization are still not fully understood (Lefevre & Bazylinski, 2013).
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59 96 Several competing hypotheses exist. Frankel *et al.* (1983) reported that $(\text{Fe}^{3+})_2\text{O}_3 \cdot 0.5\text{H}_2\text{O}$, an
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5 97 amorphous ferrihydrite, is formed first, and is then partially reduced and dehydrated to Fe₃O₄.
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7 98 Faivre *et al.* (2007) did not observe any well-ordered, crystalline precursors in a time-resolved
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9 99 Mössbauer study. They found that Fe²⁺ or Fe³⁺ was taken into the cell and converted into an
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11 100 intracellular amorphous ferrous high-spin species, predominantly located in the magnetosome
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13 101 membrane, along with ferritin. These then quickly co-precipitate to directly form Fe₃O₄ within
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15 102 magnetosome vesicles. In a recent ptychography study, Zhu *et al.* (2016) observed hematite
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17 103 (α -Fe₂O₃) in cells of *Magnetovibrio blakemorei* strain MV-1 which was interpreted to be a
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19 104 possible precursor to magnetite in the formation of magnetite magnetosomes.

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21 105 Results from various time course studies of magnetite magnetosome synthesis in MTB are
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23 106 summarized in **Supplemental Information Table S-1**. These studies all focused on either
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25 107 *Magnetospirillum magneticum* strain AMB-1 or *Magnetospirillum gryphiswaldense* strain
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27 108 MSR-1 as the model MTB for magnetite biomineralization. With some exceptions, there seems
28
29 109 to be a general consensus that:

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31 110 * a lag phase occurs before particle formation, typically in the 0.5-2 hour (h) range (Li *et al.*
32
33 111 2009).

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35 112 * crystal growth occurs in lipid bilayer membrane vesicles which are present prior to the
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37 113 visible detection of crystals (Komeli *et al.* 2004).

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39 114 * there is a period of time during which the mean size of the particles is in the
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41 115 superparamagnetic size range, significantly smaller than that of mature magnetosomes, and
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43 116 there is a broad size distribution (Firlar *et al.* 2016).

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45 117 * the particles are poorly organized in the early stages of biomineralization but become
46
47 118 arranged in chains with more or less regular spacing, after a period of time which varies
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49 119 considerably depending on the study (Heyen and Schüler 2003, Firlar *et al.* 2016), suggesting
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51 120 that the temporal evolution depends on the culturing and growth conditions (growth phase,
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53 121 O₂ concentration, temperature, etc).

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* after 24-48 h, the size and shape of individual particles and their organization in the cell approach that in magnetosomes present in stationary phase MTB grown with a major source of Fe.

* the magnetic properties of particles produced in the early stages differ greatly. Staniland *et al.* (2007), using ensemble averaged Fe L-edge X-ray magnetic circular dichroism (XMCD), stated that a full XMCD response, and thus magnetic magnetite, was detected 15 minutes (m) after Fe-depleted cells were placed in a standard growth medium containing a major source of Fe. However, Faivre *et al.* (2007) only detected Mössbauer signal indicative of magnetite 95 m after incubation with iron. Similarly, Baumgartner *et al.* (2013), using magnetically induced differential light scattering, only found a magnetic response 80 m after incubation with Fe. Baumgartner *et al.* (2013) also used ensemble averaged X-ray absorption spectroscopy (XAS) to show that Fe K-edge features typical of magnetite only appeared after ~100 m, consistent with their magnetic measurements. Li *et al.* (2009), using temperature-dependent magnetometry, detected a magnetic response characteristic of magnetite (hysteresis and Verwey transition) 28 h after the start of the time course, which, unfortunately, was their first time data point. We note that the variation in magnetic results may simply reflect the use of different methods which have different spatial, concentration and magnetic field sensitivities.

* there is an Fe(III)-rich intermediate to magnetite. While the presence of some type of Fe(III) in cells in the early stages of growth is a common observation, there is disagreement whether this is a single mineral precursor species with a unique chemical composition and structure, a mixture of species, or simply Fe(III) ions. Some report such precursors to be amorphous, others as crystalline. In some cases, this is reported as a surface α -hematite layer (Staniland *et al.* 2007, Firlar *et al.* 2019). Using XAS, Fdez-Gubieda *et al.* (2013) identified phosphorus-rich ferrihydrite as a precursor of magnetite in MSR-1 but were unable to image the precursors with high resolution transmission electron microscopy (TEM). Using K-edge XAS and extended X-ray absorption fine structure (EXAFS) and a more complete set of

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reference spectra, Baumgartner *et al.* (2013) concluded from spectra similar to those measured by Fdez-Gubieda *et al.* (2013) that a disordered ferric phosphate-like phase forms at the beginning of the time-course, which is quickly (< 30 m) converted into magnetite. Very recently, Wen *et al.* (2019) reported electron diffraction evidence of very small crystals of ϵ -Fe₂O₃ in the very early stages of their time course study of MSR-1. The lack of consistency in this aspect of the biomineralization is, at least in part, due to the use of different analytical methods, each with a specific sampling depth, spatial resolution, chemical sensitivity, and structural sensitivity.

Marcano *et al.* (2017) recently reported results from a time-course experiment which showed that the cell growth phase (lag vs. exponential vs. stationary) at the time of Fe-induction had an influence on the magnetite biomineralization process in *M. gryphiswaldense* strain MSR-1. They showed that magnetosome particle synthesis is initially slow but eventually leads to the production of larger particles and a higher cellular magnetite content for iron-depleted cultures where cells are in the exponential phase at the time of iron-induction, compared to those in the arrested growth state. This dependence on specific experimental conditions could explain the apparent lack of consistency in the results from previous time course studies.

Although numerous efforts have been made to reveal the mechanism of magnetosome biomineralization inside MTB cells, several critical questions still need to be addressed: *Are there mineral (other than magnetite) precursors formed before magnetite magnetosome formation? If they exist, can we identify and locate these precursors? How are different precursor species and/or Fe ions inside magnetosome vesicles converted to magnetite, Fe₃O₄? How do they become organized into chains with aligned magnetic moments?*

One approach to address these issues is to use spatially resolved methods to characterize the chemical and magnetic properties of MTB on an individual cell and individual particle basis throughout the process of biomineralization. Here we report results of a time course study of the biomineralization of magnetite magnetosomes in *Magnetospirillum magneticum* strain AMB-1. We used X-ray absorption spectroscopy (XAS) and XMCD measured at the Fe L₃-edge by

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5 176 scanning transmission X-ray microscopy (STXM) to characterize cellular interior and individual
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7 177 magnetosomes in cells at various time points after initiation of biomineralization. XAS can
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9 178 identify different Fe species and thus identify precursor particles and possibly soluble Fe species
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11 179 (Zhu *et al.* 2016). XAS and XMCD can be used to monitor how the ratio of Fe(II) to Fe(III)
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13 180 changes as the biomineralization of Fe₃O₄ proceeds. The power of our approach over most
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15 181 earlier time course studies is the ability to examine and spectroscopically analyze individual
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17 182 particles within a single cell. Our strategy was to start with cells grown in the absence of Fe
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19 183 which do not contain magnetosomes, and then to initiate magnetosome biomineralization by
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21 184 inoculating these cells into standard Fe-containing culture medium. We then sampled at various
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23 185 times during a 48 h period after the start of the time course, and subsequently measured TEM
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25 186 and STXM from those samples in order to investigate magnetosome biomineralization and chain
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27 187 formation over time.

28 29 188 30 189 31 32 190 **2. Experimental**

33 191 34 35 192 *2.1 Sample preparation*

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37 193 Cells of *M. magneticum* strain AMB-1 were initially grown in a Fe-rich liquid medium
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39 194 under controlled microaerobic conditions with 1.5% O₂ in the headspace of the culture, as
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41 195 previously described (Le Nagard *et al.* 2018). The growth medium contained, per liter: 1.0 mL
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43 196 modified Wolfe's mineral elixir (Wolin *et al.*, 1963, Bazylinski *et al.*, 2000), 0.1 g KH₂PO₄, 0.15
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45 197 g MgSO₄·7H₂O, 2.38 g (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer),
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47 198 0.34 g NaNO₃, 0.1 g yeast extract (BD 210929), 3.0 g Becton Dickinson (BD) Bacto™ Soytone
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49 199 (BD 243620), 4.35 mL potassium lactate (60% solution) and 5 mL Fe (III) citrate 10 mM stock
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51 200 solution.

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53 201 Several weeks prior to starting a time course experiment, cells were grown in a Fe-depleted
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55 202 liquid growth medium similar to the Fe-rich growth medium except that the amount of BD
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57 203 Bacto™ Soytone was reduced to 1.0 g/L and the Fe(III) citrate and mineral solutions and the
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59 204 yeast extract were omitted. For the first set of samples (batch A, November 2017), cells were

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5 205 grown in the Fe-depleted medium with 5% O₂ in the headspace. The increased O₂ concentration
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7 206 led to a quicker decrease in the number of magnetosomes per cell compared to cultures grown in
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9 207 Fe-depleted conditions with 1.5% O₂ in the headspace. It took 5 successive inoculations into
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11 208 fresh, Fe-depleted medium over a 2-week period to achieve cultures in which standard measures
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13 209 of a magnetic population – hanging drop and reflectivity modulation over a magnetic stirrer –
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15 210 indicated that most cells had lost their magnetism. In addition, transmission electron microscopy
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17 211 (TEM) showed most cells did not contain particles, although some had 1 or 2 particles. For batch
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19 212 B, the O₂ concentration in the headspace was increased to 20%, which caused cells in the culture
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21 213 to grow more vigorously, and to become non-magnetic even more quickly than at 5% O₂.
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23 214 Respectively, >10 and 7 stages of depletion were performed for the batch A and B samples. At
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25 215 each stage, 1 mL of the (n-1) stage culture was inoculated into 60 mL of fresh Fe-depleted
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27 216 medium and incubated at 32 °C for 3-4 days in order to reach the stationary phase, before
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29 217 performing the next transfer. The magnetism of the culture was assessed at each stage using the
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31 218 hanging drop test, in which the response of the cells to an external magnetic field is studied using
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33 219 optical microscopy (Le Nagard *et al.*, 2018b). TEM was also used to estimate the number of
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35 220 particles per cell. For both batch A and batch B, there was no observable change in the hanging
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37 221 drop test and in TEM analysis results after 5-6 depletion stages.

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40 222 The time course experiment was initiated by inoculating 30 mL of the Fe-depleted culture
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42 223 into 30 mL of the Fe-rich medium under anaerobic conditions. O₂ was immediately added to the
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44 224 culture after inoculation to obtain a concentration of 1.5% O₂ in the headspace of the culture.
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46 225 Samples were then removed from the culture at intervals of 0, 10, and 30 min (m) and 1, 2, 4,
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48 226 5.7, 8, 12, 24, and 48 hours (h). Immediately after collecting a culture sample, the extracted
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50 227 aliquot was placed into a centrifuge vial and heated at 60 °C for 10 m to kill the cells and stop
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52 228 magnetosome biomineralization (Cazares *et al.* 2015). These samples were then stored at 4 °C
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54 229 until they were prepared for TEM and STXM.

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56 230 For the batch A samples, ~2 µl were drop cast on to formvar coated 3 mm TEM grids. A
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58 231 washing step was not applied, and as a consequence, most of the cells were encrusted with
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5 232 residual salts from the culture medium. For the batch B samples, 3 different sample mounting
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7 233 approaches were used. Immediately after heat sterilizing the cells at each time point during the
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9 234 time course, about 2 μ l were drop cast on to C-flat 3 mm TEM grids
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11 235 (<https://www.protochips.com/products/c-flat/>). After investigation by TEM it was found that
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13 236 there were still some residual salts (but much less than in the batch A samples) so a new set of
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15 237 batch B samples for STXM was prepared using glutaraldehyde fixation (Chao & Zhang 2011)
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17 238 followed by multiple washings in distilled deionized water and centrifuge separations. This has
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19 239 been shown to result in samples free of salt precipitates, with cells retaining the particles in their
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21 240 as-grown state (Le Nagard *et al* 2018a). The glutaraldehyde-fixed material was drop cast on
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23 241 silicon nitride windows (Norcada) and formvar coated TEM grids.
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26 243 *2.2 Scanning Transmission X-ray microscopy (STXM)*

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29 244 STXM analyses were performed at HERMES, the soft X-ray spectromicroscopy beamline at
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31 245 SOLEIL (St. Aubin, France) (Belkhou *et al*, 2015, Swaraj *et al.*, 2017). STXM methodology has
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33 246 been described in detail previously (Hitchcock 2012, Hitchcock 2015). Briefly, monochromated
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35 247 X-rays are focused to a \sim 30 nm spot by a Fresnel zone plate. The sample is positioned at the
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37 248 focal point and x-y raster scanned while recording the intensity of the transmitted X-rays. After
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39 249 locating cells of interest, aided by optical imaging and, in some cases, TEM, STXM images were
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41 250 measured at a sequence of photon energies (a stack). In all cases, the region measured contained
42
43 251 a suitable area free of cells to measure the incident flux (I_0), which is needed to convert
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45 252 transmitted intensities to optical density (OD). Circular right (CR) polarized light was used
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47 253 without any applied magnetic field. For some samples measurements were performed with both
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49 254 helicities – i.e. both circular left (CR) and circular right (CL) polarized light. XMCD maps were
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51 255 then generated from the difference of OD-converted CR- and CL-images at 708.2 eV - the
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53 256 energy of strongest X-ray magnetic circular dichroism (XMCD) signal for magnetite (Goering *et*
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55 257 *al.* 2007, Zhu *et al.* 2015). XMCD spectra are the (CR – CL) difference, recorded at all energies
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57 258 across the Fe L_3 edge. STXM studies of the batch A samples were performed in November 2017
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59 259 with the sample tilted at 32° relative to the focal plane. For the batch B samples, XAS and
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5 260 XMCD were measured in December 2018, using both 0° tilt (X-ray beam direction normal to the
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7 261 sample plane) and 30° tilt (sample grid tilted at 30° relative to the focal plane).

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10 263 2.3 Data analysis – STXM, XAS & XMCD

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12 264 Most of the results were obtained using XAS stacks - a set of images measured over a
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14 265 sequence of photon energies in the Fe L₃ region (700 – 717 eV). After image alignment and
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16 266 conversion of the transmission signal to OD, the many-energy stacks were further processed by
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18 267 extracting internal reference spectra from unique morphologies such as the particles and the
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20 268 cytoplasm, then fitting the stack to those reference spectra in order to generate component maps.
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22 269 In some cases, the component maps are presented as color coded composites in which the signal
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24 270 of each component is scaled to fill its color scale (so called rescaled, rather than absolute color
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26 271 composites) (Hitchcock, 2012). In cases where both CR and CL multi-energy stacks were
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28 272 acquired, these were combined into a single file prior to alignment and OD conversion, and then
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30 273 separated to allow generation of XMCD stacks from the difference (CR-CL) which allowed
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32 274 extraction of the magnetic signal from individual particles. All STXM data analysis was
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34 275 performed using aXis2000 (<http://unicorn.mcmaster.ca/aXis2000.html>).

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36
37 276 In this work, in contrast to typical XMCD measurements in materials science, the intrinsic
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39 277 magnetic properties of a sample are measured without using any applied magnetic field. There
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41 278 are strict geometric requirements to measure magnetization by XMCD. As outlined in **Fig. 1**, the
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43 279 magnitude of the XMCD signal is determined by the dot product of the magnetization vector of
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45 280 the sample and the polarization vector of the photon. The polarization vector of circularly
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47 281 polarized photons points either forward (CR) or backward (CL) along the X-ray propagation
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49 282 direction. In order to have a measurable XMCD signal there must be a non-zero projection of the
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51 283 magnetization vector of the sample along the photon polarization. The magnetization vector of
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53 284 chains of magnetosomes in magnetotactic bacteria grown in high-Fe culture is typically along the
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55 285 cell axis. Thus, a cell lying flat on its support should be positioned such that the long axis of the
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57 286 cell is horizontal and the sample is tilted out of the plane normal to the X-ray propagation
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59 287 direction. We typically use a polar tilt angle of ~30° for which the projection of an in-plane,

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288 horizontally oriented magnetic moment is 50%. Since monitoring the development of the chains
289 and their magnetic alignment is the goal of this project, we have made measurements at both 0°
290 and 30° tilt angles. Any XMCD signal detected with the sample at a tilt angle of 0° indicates
291 there is an out-of plane component to the magnetization (i.e. a component orthogonal to the grid
292 on which the cell is deposited).

294 *2.4 Data analysis –particle size distributions from statistical evaluation of TEM images*

295 The average number of particles and their typical size in cells of the glutaraldehyde-fixed
296 Batch B samples were determined by TEM. Individual cells were randomly selected on the grids
297 and imaged at 40,000x – 60,000x magnification. This study was performed after the STXM
298 measurements, to avoid damaging the samples with high magnification TEM. 20 cells were
299 imaged per sample, and a minimum of N=18 cells in each sample were subjected to detailed
300 image analysis. Particles were counted and measured manually. Large variations in the contrast
301 of the particles (size dependency and inhomogeneous background) made it difficult to use an
302 automated procedure. The oval selection tool of the ImageJ software was used to draw a shape
303 that best matched the projection of each particle in the focal plane. The surface area of that
304 projection was measured and converted to a diameter, assuming the particles to be spherical.
305 There is significant uncertainty in the measurement of particles smaller than 10 nm, due to
306 insufficient spatial resolution at the magnifications used in this study. These particles were
307 excluded from the final results. After excluding these very small particles, independent analyses
308 of the 24 h sample performed by two different experimentalists differed in the average number
309 and size of particles by only 7% and 2% respectively.

311 **3. Results**

312 *3.1 Overview of the time course samples*

313 **Figure 2a** shows STXM maps of the Fe-containing particles in one cell from each time
314 course point for the batch B samples. Early in the time course (10 m, 30 m), cells contained no or

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5 315 just a few particles. The average Fe L₃ spectrum of all the particles within each cell in **Fig. 2a** are
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7 316 plotted in **Fig. 2b**. Over the time course there is a general increase in the intensity of the Fe L₃
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9 317 signal, and some shape changes, which are discussed in a later section. A detailed description of
10
11 318 the particle size and number per cell at each time point in the batch B time course, as measured
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13 319 by TEM is presented in section 3.2. In terms of particle numbers, there were no significant
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15 320 differences between the samples taken at 10 m and 30 m after the start of the experiment,
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17 321 followed by an increase in the number of particles per cell in subsequent time points. Cells from
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19 322 the 1 h and 2 h sample exhibited only a few particles. A transition occurred in cells of the 4 h and
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21 323 5.7 h samples, in which many more particles were present and chains started to appear. Cells in
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23 324 samples taken at later times contained relatively large magnetite particles assembled in chains of
24
25 325 varying lengths. The average number of particles per cell and extent of chain formation at 48 h
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27 326 were indistinguishable from stationary phase cells grown in Fe-rich media.

29
30 327 **Supplemental figure S-1** shows STXM images of cells from **batch A** at each time point. In
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32 328 the batch A experiments, particles appeared after 2 h in some cells. XAS spectra at the Fe L₃
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34 329 edge of particles from these experiments are presented in **Supplemental Fig. S-1g**. A systematic
35
36 330 increase in the X-ray absorption at ~710 eV of individual particles with time was observed. The
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38 331 images and STXM-XAS results showed that a mixture of large and small particles was present in
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40 332 cells from the 4 h sample. Only a few isolated particles were observed in the <2 h samples. By
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42 333 48 h, STXM images of the cells examined were indistinguishable from STXM images of
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44 334 stationary phase AMB-1 cells cultured in Fe-rich medium, both in terms of imaging (**Fig. S-1f**),
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46 335 and Fe L₃ spectra (**Fig. S-1g**). On a qualitative basis, the trends seen in batch A and B are
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48 336 similar.

52 338 *3.2 Statistics of particle size and number of particles per cell*

54 339 A stacked bar chart of the size distribution of particles per cell at each time point, as
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56 340 determined by TEM, is shown in **Figure 3a**. The change over the time course in numbers within
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58 341 each of 4 particle size ranges is presented on a log(time) plot in **Fig. 3b**. The numerical data is

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5 342 presented in **Supplemental Table S-2** while **Fig. S-2** shows histograms of the size distribution at
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7 343 each time point. Fig. 3b indicates there is a 30 m lag phase followed by growth of particles. This
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9 344 is consistent with recent results on AMB-1 reported by Amor *et al.* (2018, 2019) in which they
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11 345 find evidence for a similar lag phase, which they suggest correlates with the build-up of a pool of
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13 346 intracellular iron, distinct from the magnetosomes. The average number of particles per cell was
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15 347 about 13 after 2 h. Many of these particles were very small, probably below the spatial resolution
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17 348 of STXM. Particle counts per cell for AMB-1 cells grown in an Fe-rich medium reported in the
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19 349 literature range from 11 (Wang *et al.* 2013) to 20 (Tanaka *et al.* 2008).
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21 350 22 23 351 *3.3 Reference STXM – XAS/XMCD of mature AMB-1 magnetosomes*

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25 352 **Figure 4** presents a STXM image of a AMB-1 cell taken from stationary phase culture
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27 353 grown in Fe-rich media, along with magnet maps, and the XAS and XMCD spectra of the
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29 354 magnetosomes. The sample was tilted at 30° relative to the X-ray propagation direction and CR
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31 355 was used. **Figure 4a** is the average of 36 OD images from 704 to 717 eV while **Fig. 4b** is a color
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33 356 coded composite of component maps derived by fitting the Fe L₃ stack to the spectra presented in
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35 357 **Fig. 4c**. The fitting identifies the magnetosomes with a parallel (red) and those with an
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37 358 antiparallel (blue) alignment of their magnetic moment with respect to the circular polarization
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39 359 vector of the photon (see **Fig. 1**). In **Fig. 4c** the dots are the average spectra of all the
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41 360 magnetosomes with parallel (red) or anti-parallel (blue) magnetic alignment, while the solid lines
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43 361 are spectra reported for synthetic magnetite (Goering *et al.* 2007) with their intensity scale
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45 362 adjusted to match the OD of the AMB-1 magnetosome spectra. Although there is an offset, the
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47 363 min-max magnitude of the XMCD signal in the AMB-1 magnetosomes is the same as that in
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49 364 magnetically-saturated magnetite, indicating the magnetization of the magnetosomes is fully
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51 365 saturated and the magnetic moment is oriented in the plane of the sample. A similar approach
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53 366 was used to demonstrate that the magnetism of magnetosome in cells of *Magnetovibrio*
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56 367 *blakemorei* strain MV1 is saturated (Kalirai *et al.* 2016). We note that normally CR and CL
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58 368 spectra are normalized to the edge jump and presented as an asymmetry ratio. However, there
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5 369 was not sufficient beam time to allow measurement of full Fe L₂₃ spectra for this type of
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7 370 normalization.

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9 371 **Figure S-3** presents results from XAS-XMCD studies of two AMB-1 cells from the 48 h
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11 372 time point in batch B, measured using CR with the sample tilted 30° relative to the X-ray beam.
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13 373 In this case, in contrast to Fig. 4, the magnetic polarity of each magnetosome in an individual cell
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15 374 is the same, but the magnetic moment of the chains in the left cell are pointing opposite to that in
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17 375 the right cell, as shown by the color coded composite of signals from an XMCD stack map (**Fig**
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19 376 **S-3a**). Full Fe L₃ stacks of the left cell were recorded using both CR and CL polarization, in
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21 377 order to obtain the XAS and XMCD signals. **Fig. S-3b** compares the spectra of magnetosome
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23 378 particles from the 48 h sample recorded with both circular polarizations, in comparison to the
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25 379 data from **Fig. 4** and that of magnetite (Goering *et al.* 2007). The spectrum of the cytoplasm (also
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27 380 plotted in **Fig. S-3b**) does not have any Fe L₃ signal. The CR stack was fit to the parallel and
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29 381 anti-parallel reference spectra to generate component maps, which are presented as a color-coded
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31 382 composite in **Fig. S-3c**. Within each color the signal for that component has been rescaled to the
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33 383 full 0-255 scale. At the particles the parallel signal is strong while the anti-parallel signal is
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35 384 weak, possibly just noise. The XMCD signal (**Fig. S-3b**) is strong, similar in intensity to that of
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37 385 an AMB-1 cell taken from stationary phase culture grown in Fe-rich media (**Fig. 4**), and to that
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39 386 of XMCD of magnetically saturated magnetite (Goering *et al.* 2007), scaled *0.5 to account for
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41 387 the experimental geometry (tilt angle of 30°).
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45 389 *3.4 Time-dependent STXM-XAS Study of the Biomineralization Process*

46 390 Typically, 2 or 3 cells at each time point of the batch B time course were measured in detail.
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48 391 In each case, there were only a few scattered individual particles present in cells up to 1 h.
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50 392 Disordered chains and larger particles appear in cells from the 2 h sample. Well-developed
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52 393 chains similar to those in cells from stationary phase culture grown in Fe-rich medium were
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54 394 observed in cells from the 24 h and 48 h samples (see **Fig. 2a**). Cells from the 8 h time point of
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56 395 batch A, and the 5.7 h and 24 h time points of batch B were particularly interesting and are
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58 396 highlighted in the next sections.
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5 397 A detailed analysis of a cell from the 8 h sample in batch A is presented in **Fig. 5. Figure 5a**
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7 398 shows that this cell contained one chain with 6 particles (left chain, red), a second chain in the
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9 399 middle with 3 small particles (middle chain, cyan) which is interpreted as a “growing” chain, and
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11 400 a third chain at the right with 3 very small particles which are interpreted as particles in the early
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13 401 stages of growth (precursor, green). In addition, there is a single large magnetosome (blue).
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15 402 **Figure 5b** compares the background subtracted Fe L₃ spectra of the large magnetosome, the left
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17 403 chain, the middle (“growing”) chain and the chain of precursor particles from this cell, on an
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19 404 absolute OD scale. **Fig. 5c** plots the same spectra on a constant intensity format, with
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21 405 comparisons to reference spectra of FeCl₃ (Nagasaka, *et al.* 2013) and parallel and antiparallel
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23 406 circular dichroic spectra of synthetic magnetite (Goering *et al.* 2007). The spectrum of the large
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25 407 magnetosome is similar to that of magnetite with antiparallel magnetic moment orientation
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27 408 relative to the CR light. The spectrum of the left chain is similar to that of magnetite with parallel
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29 409 magnetic moment orientation relative to the CR light. In the spectrum of the particles in the
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31 410 middle chain (cyan) the signal at 708.4 eV is significantly more intense than that of magnetite.
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33 411 This is consistent with α -hematite (Zhu *et al.* 2015). **Supplemental figure S-4** plots the Fe L₃
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35 412 spectra of several of the particles from early time course samples in comparison with the spectra
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37 413 of α -hematite (Zhu *et al.* 2015), ferrihydrite (Joshi *et al.* 2018), FeCl₃ (Nagasaka, *et al.* 2013)
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39 414 and Fe(III) phosphate (Miot *et al.* 2009). While the differences are subtle, in the spectrum of the
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41 415 particles of the 5h 40 m sample, the position and, especially, the intensity of the 708.4 eV signal
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43 416 relative to the main peak at 709.8 eV is close to that of α -hematite. However the spectrum of the
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45 417 particles in the precursor chain of the 8 h sample is more similar to the Fe L₃ spectra of
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47 418 ferrihydrite (Joshi *et al.* 2018) or FeCl₃ (Nagasaka, *et al.* 2013). The spectrum of the particles in
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49 419 the “growing” chain is intermediate between the two, although closer to the spectrum of
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51 420 magnetite.
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54 421 To further focus on results from specific time course points, the STXM-XMCD results from
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56 422 cells taken at the 5.7 h (5 h 40 m) and 24 h time points are discussed below. In addition, results
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58 423 and discussion of the 4 h sample from batch B is given in the Supplemental Information. **Figure**
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5 424 **6** presents results from the 5.7 h sample (batch B) measured with the sample tilted at both 0° and
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7 425 30° relative to the X-ray beam. In this case the cell examined was drop cast directly from the 5.7
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9 426 h solution onto a C-flat grid, without washing. Despite that, the cell was very clean, without
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11 427 adhering salts from the culture. **Fig. 6a** and **6b** present TEM images of the same cell, measured
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13 428 after the STXM measurements. There are clear signs of carbon deposition in the rectangle
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15 429 irradiated during the measurement (**Fig. 6a**). **Figure 6c** shows the sum of all images in an Fe L_3
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17 430 stack map, measured with the sample normal to the beam (tilt = 0°). **Figure 6d** represents an
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19 431 XMCD map at 708.2 eV measured at tilt = 0° showing that a number of the particles exhibit
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21 432 out-of-plane magnetization. **Figure 6e** shows an XMCD map of the same set of particles,
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23 433 measured at tilt = 30° . The XMCD signal intensity has significantly increased and all but the two
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25 434 particles at the ends of the disorganized chain have the same magnetic moment direction, while
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27 435 the magnetic moment of the particles at the end is in the opposite direction. The magnitude of the
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29 436 XMCD signal of the leftmost particle is of opposite sign and twice as strong at 0° (-0.08) than at
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31 437 30° tilt (+0.04). **Figure 6f** presents XAS measured with CR and CL polarization and the resulting
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33 438 XMCD spectrum, averaged over all the particles in **Fig. 6e** which have a negative XMCD signal
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35 439 (dark). A clear XMCD signal was obtained for this cell and the positioning of the particles was
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37 440 such that chain formation appears to be occurring (**Fig. 6e**).

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40 441 **Figure 7** plots the Fe L_3 spectra of five different iron-containing regions of an AMB-1 cell
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42 442 from the 24 h time point (batch B). Spectral variability in the Fe L_3 stack was assessed by careful
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44 443 examination of the Fe L_3 spectra of the different morphologies. Five distinct Fe L_3 spectra were
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46 444 detected, which when used to fit the stack, resulted in a high quality fit with very small residuals
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48 445 and clear, spatially localized component maps. A multivariate statistical analysis using the
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50 446 PCA_GUI code (Lerotic *et al.*, 2005) supported the existence of 6 or 7 independent components,
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52 447 consistent with the analysis presented here. These spectra were used to derive the results
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54 448 presented in **Fig. 8**, which includes component maps from the 5-component fit to the Fe L_3 stack,
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56 449 and a color coded composite map for cells of the 24 h sample (glutaraldehyde fixed). **Fig. 8a**
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58 450 shows an OD-converted image of the full cell. **Fig. 8b** shows the average of all images in a

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5 451 60-energy Fe L₃ stack measured on the right part of the cell, which has well-developed
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7 452 magnetosome chains. The component maps, i.e., maps of the areas with spectra like one of the 5
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9 453 reference spectra, are displayed in **Fig. 8c-g**. A color coded composite of the parallel XMCD
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11 454 (red), cytoplasm (green) and antiparallel XMCD (blue) maps is presented in **Fig. 8h**. A notable
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13 455 feature of this cell is a well-defined ‘ring’ or rim around the central, expanded part of the cell.
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15 456 While the spectrum of the rim has a large non-Fe component, it also has a broad Fe L₃ signal
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17 457 which is at higher energy than the Fe signal of the weak but statistically significant Fe L₃ signal
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19 458 in the cytoplasm of the cell. Finally, one of the particles has a strong Fe(III) signal, different
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21 459 from the small particles in the 5.7 h and 8 h cells which show enhanced 708.4 eV signal. That
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23 460 Fe(III) particle did not exhibit any XMCD (compare **Fig. 8d** and **Fig. 8e, 8f**).
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27 462 **Fig. 9** presents the circular dichroic dependent Fe L₃ XAS spectra and the derived XMCD
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29 463 signal from the 10 magnetosomes in the 24 h cell (**Fig. 8e**). The intensity scale is that for the
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31 464 optical density of the AMB-1 24 h cell data. **Fig. 9** also presents the parallel and antiparallel
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33 465 spectra of magnetite reported by Goering *et al.* 2007, scaled to match the magnitude of the signal
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35 466 from the AMB-1 cell. After correcting by a factor of 2 for the projection of the 30° tilted sample
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37 467 onto the spin vector of the X-rays, the magnitudes of the XMCD signal of pure magnetite and
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39 468 that of the magnetosomes are very similar. This indicates that, after 24 h of the time course, the
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41 469 particles are magnetite and their magnetization is fully saturated.
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44 470 **Supplemental Figure S-6** compares color-coded composites of the Fe-rich components
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46 471 obtained from two different models for mapping the chemical components of the 24 h time point
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48 472 sample from batch B from the Fe L₃ stack. **Fig. S-6a** was obtained from the stack measured with
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50 473 CL using only 4 components (spectra of regions with XMCD+, XMCD-, cytoplasm and Fe(III),
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52 474 without an explicit ‘rim’ signal) while **Fig. S-6b** was obtained from the stack measured with CR
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54 475 using the 5 components displayed in **Fig. 7**. If a specific spectrum of the ‘rim’ is not included
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56 476 then the area corresponding to the ‘rim’ is fit with the nominal CL component. However, if the
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58 477 ‘rim’ spectrum is included in the fit then the rim signal fits to both the rim and regions in the
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5 478 vicinity of the magnetosomes. The model including an explicit rim spectrum is better.

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7 479 **Supplemental Figure S-7a-c** are images of the XMCD stack for the 24 h sample. **Figure S-7d**
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9 480 displays the XMCD spectra of the magnetosomes while **Fig. S-7e** presents the XAS spectrum of
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11 481 the rim. The XMCD spectrum of the 'rim', plotted in **Fig. S-7d**, showing there is no detectable
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13 482 XMCD in the 'rim' region.

14 15 483 16 17 484 18 19 485 **4. Discussion**

20 486 *4.1 What we have learnt?*

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23 487 Our results clearly show that small numbers of iron oxide particles were generated in cells of
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25 488 *M. magneticum* strain AMB-1 grown without a major source of iron, relatively early (<30 m)
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27 489 after the major source of iron, Fe(III) citrate, was introduced. These particles were significantly
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29 490 smaller than those in magnetosomes in cells of cultures incubated for longer periods. The spectra
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31 491 of the particles in cells taken at 10 m, 30 m, 1 h, and 2 h showed a signal at 708.4 eV that is
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33 492 stronger than that of magnetite, indicating contributions from an Fe(III)-rich precursor. The
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35 493 samples on TEM grids or SiN_x windows were stored under dry N₂ from the time of preparation
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37 494 to studies at SOLEIL. It has also been shown that magnetosome magnetite crystals which are
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39 495 surrounded by their lipid membrane oxidize very slowly even when exposed to air for many
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41 496 months (Zhu *et al.* 2015). Thus we conclude that the additional oxidized character of the smaller
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43 497 particles in the early time points is a characteristic of the biomineralization of magnetite in *M.*
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45 498 *magneticum*, and thus those particles can be considered precursors to magnetite. XMCD maps
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47 499 (CR and CL at 708.2 eV, the energy of strongest XMCD contrast) and/or Fe L₃ stacks were
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50 500 measured for 5 of the 9 samples.

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52 501 One concern in interpreting these results is the question of the degree to which particles
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54 502 observed at early time points might have been magnetite magnetosomes in cells inherited from
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56 503 the original inoculum (which was grown in Fe-rich medium) or possibly even iron oxide
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58 504 particles grown in Fe-depleted medium due to the presence of the very low concentration of Fe
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60 505 required for cell growth, possibly present in BactoTM Soytone. The 10 m samples in both batch A

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5 506 and B contained a few cells with one particle that was quite large (40-50 nm) suggesting that it
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7 507 could be a residual magnetosome from the original pre-Fe-depletion culture, despite many cycles
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9 508 of re-inoculating in Fe-depleted medium to dilute the original cells and reduce the number of
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11 509 magnetosomes per cell by allowing the cells to divide a significant number of times. Due to this
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13 510 ambiguity we have placed greater reliance on samples at later time points (≥ 1 h) in developing
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15 511 our interpretation of these results.

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17 512 In cells from the 4 h sample from batch B, 1 out of 5 particles showed an XMCD signal with
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19 513 the sample at 0° tilt. (**Fig. S-5**). The 4 small particles in the same cell did not exhibit a
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21 514 measurable XMCD signal. Magnetosome crystals in cells grown for many generations in Fe-rich
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23 515 growth medium only exhibit XMCD if the cell is \sim horizontal and tilted at 30° to the beam
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25 516 propagation direction (Lam *et al.* 2010). The observation of a strong out-of-plane magnetization
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27 517 is evidence that the magnetic particle was recently biomineralized. In the absence of chain
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29 518 formation and presumably magnetic dipolar interaction with adjacent magnetite particles, the
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31 519 magnetic moment could point in any direction. If the particle-particle magnetic interaction
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33 520 between pre-existing magnetosome(s) and a newly biomineralized magnetosome(s) is not strong
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35 521 enough to align the magnetic vector of the new one, the magnetic orientation of the newly
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37 522 forming magnetosome would be determined by external factors such as the local magnetic field
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39 523 of the earth at the time of particle formation. Winklhofer *et al.* (2007) reported that the degree of
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41 524 preferential alignment was 0.54:0.46 at a magnetic field of 0.26 G (about that of the earth in
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43 525 Hamilton, ON where the time course samples were grown) and rose only to 0.58:0.42 at a field
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45 526 of 0.50 G.

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47 527 It should be noted that the visual gap between magnetosomes with opposite magnetic
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49 528 polarities (e.g. **Fig. 6e**) might not represent the real distance between these magnetosomes, since
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51 529 2D projection images can mask the actual 3D distance information. In this regard, XMCD
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53 530 tomography, which can map the magnetization strength and direction of individual particles in
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55 531 3D, of a cell displaying both in-plane and out-of-plane magnetization might provide additional
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57 532 insight into how MTB achieve the well organized and well correlated magnetism of adjacent
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59 533 magnetosomes. Electron tomography was very useful in showing that lipid vesicles in which
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4 534 magnetosomes are formed bud from the exterior cell membrane (Komeli *et al.*2004). Cells from
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6 535 the 5.7 h batch B contained numerous particles (>12 /cell on average), the crystals in some of
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8 536 which exhibited XMCD at 0° with opposing moment orientations (**Fig. 6**). When the same
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10 537 sample was measured at 30°, an XMCD signal was observed for the majority of the particles,
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12 538 indicating a chain with a more organized magnetic structure was being formed. In cells from the
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14 539 24 and 48 h samples, the XAS spectra measured at 30° and the magnitude of the derived XMCD
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16 540 signal of the magnetosomes was similar to that of synthetic magnetite (Goering *et al.* 2007) (**Fig.**
17 541 **S-3b**).

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19 542 Iron oxide particles were observed in cells from the 1 – 8 h time points which had a
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21 543 measurably smaller diameter and weaker Fe L₃ signal than that of mature paticles found in
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23 544 AMB-1 cells, and typically a stronger signal at 708.4 eV than is expected for magnetite. These
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25 545 particles are interpreted as growing particles and thus their properties are providing direct
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27 546 information about the biomineralization mechanism. The stronger 708.4 eV signal is evidence
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29 547 for a higher Fe(III) character than that of magnetite. The comparison of the position and intensity
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31 548 of the 708.4 eV signal relative to the main 709.8 eV peak with that in other Fe(III) compounds
32 549 (**Fig. S-4**), suggests that α -hematite (Zhu *et al.* 2015) is a possible intermediate in the
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34 550 development of mature magnetosomes. We note that α -hematite was also found in previous time
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36 551 course studies by Baumgartner *et al.* (2013) and Firlar *et al.* (2016, 2019), although both of those
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38 552 studies, along with that by Fdez-Gubieda *et al.* (2013), concluded that a phosphate-rich ferric
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40 553 hydroxide phase was involved. We also find a precursor signal consistent with FeCl₃ and/or
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42 554 ferrihydrite, in agreement with the main conclusions of Baumgartner *et al.* (2013) and
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44 555 Fdez-Gubieda *et al.* (2013). A better understanding of the role of these two species in the
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46 556 biomineralization pathway is now required. We note that the statistical quality of the Fe L₃
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48 557 spectra from the particles in the earlier time points is limited. In addition the spectra are averaged
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50 558 over several particles, which may be at different stages of development and thus might have
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52 559 different speciation. Finally, we did not have enough beam time to adequately sample times
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54 560 below 1 hour and thus our results are silent in that early period, where Wen *et al.* (2019) find
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56 561 evidence for ϵ -Fe₂O₃ from single particle electron diffraction.

56 562 A striking observation in this study is that small particles are formed at multiple places in the
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58 563 cell, not necessarily in chains, and with seemingly no detectable ferromagnetism, or with
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60 564 randomly oriented magnetic moments. The observation of these putative precursor particles in
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4 565 different cell locations and at different times time course points suggests that magnetosomes are
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6 566 not biomineralized simultaneously. Regarding the appearance of precursors at multiple sites
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8 567 within the cell, Scheffel *et al.* (2006) have shown that magnetite biomineralization is initiated at
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10 568 multiple discrete sites throughout the length of *Magnetospirillum* cells. Thus mature
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12 569 magnetosomes and magnetosome precursors can coexist in the same cell since some may grow
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14 570 faster than others. The acidic protein MamJ was shown to be responsible for anchoring the
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16 571 magnetosome to a filamentous structure now recognized as the protein MamK (Arakaki *et al.*
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18 572 2008). As for the lack of ferromagnetism observed in these precursors, this could be because
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20 573 these small particles are not (yet) magnetite and thus not magnetic at room temperature.
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22 574 Alternatively they could be smaller than the ~30 nm super-paramagnetic limit in magnetite
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24 575 (Dunlop 1973), and thus subject to thermally induced re-orientation of their magnetic moment. A
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26 576 third explanation could be that the early particles are Fe-rich polyphosphates formed in the
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28 577 cytoplasm, as was found in some other bacterial systems (Miot *et al.* 2009). STXM
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30 578 measurements at the P 2p or P 1s edge could be used to investigate this last possibility.

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32 579 In the intermediate time points (4 – 8 h) chain formation is increasingly seen, and a larger
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34 580 fraction of the particles are located in chains with an in-plane magnetic moment (e.g. the 5.7 h
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36 581 batch B sample, **Fig. 6**, and the 8 h batch A sample, **Fig. 5**). It is only in the 24 h and 48 h
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38 582 samples that the size, spectroscopy, magnetic moment magnitude and orientation match that of
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40 583 magnetosome chains in mature AMB-1 cells grown in Fe-rich culture.

41 584 From a microbiological point of view, this study shows that *M. magneticum* strain AMB-1
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43 585 will grow for extended periods of time when several nutrient sources usually added to the growth
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45 586 medium are omitted or added in significantly lower concentration – in this case, yeast extract,
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47 587 Bacto™ Soytone, mineral solution in addition to Fe(III) citrate, the major Fe source of the
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49 588 normal growth medium. Increasing the O₂ concentration does not prevent growth either, showing
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51 589 that *M. magneticum* can adapt to a relatively wide range of conditions, from microaerobic to
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53 590 fully oxic conditions. When cultured at high concentrations of O₂ and low concentrations of Fe
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55 591 for extended periods of time, the cells biomineralize only a few crystals and grow as vigorously
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57 592 as in normal media. These findings raise important questions regarding the magneto-aerotaxis
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59 593 model for the function of magnetosomes in magnetotactic bacteria, at least for *M. magneticum*. If

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5 594 the cells grow in presence of high concentrations of O₂ expending energy to produce
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7 595 magnetosomes no longer seems advantageous if their only role is to help the cells locate the OAI
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9 596 more efficiently. Finally, as seen during this and other time course experiments, cells start
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11 597 biomineralizing new crystals quite quickly once placed under low O₂ and relatively high Fe
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13 598 concentrations. This shows that the machinery used to perform biomineralization is conserved
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15 599 during periods of Fe starvation and that it is quickly reactivated when the environmental
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17 600 conditions are suitable.

19 601 20 21 602 *4.2 Suggestions for future studies*

23 603 While these results contribute to our understanding of magnetosome biomineralization, they
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25 604 are still ambiguous with regard to the identity of the Fe(III)-rich precursor species. Many studies
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27 605 have shown that it is possible to use XAS and XMCD to differentiate among the candidate
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29 606 species (α -hematite, Fe phosphates, ferrihydrite). However better statistics than we were able to
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31 607 achieve in this study are required. In part this is a function of limited experimental time at the
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33 608 synchrotron STXM, and in part a consequence of limited spatial resolution. The former issue can
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35 609 be addressed by focusing on just a few representative cells in the 1-6 h time course points where
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37 610 the evolution of the chemistry and organization is occurring. More precise spectra could then be
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39 611 fit to a linear combination of the 2 contributions and used to extract the ratio of the precursor and
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41 612 magnetite components as a function of time. Higher spatial resolution can be achieved by using
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43 613 the emerging method of soft X-ray ptychography (Thibault *et al.* 2014, Hitchcock 2015) which
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45 614 has already been applied to MV-1 MTB (Zhu *et al.* 2016). In addition to XAS and XMCD in 2D
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47 615 projection, it would be beneficial to perform XMCD tomography in order to locate the spatial
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49 616 orientation of the magnetic moment of particles in cells like the 5.7 h sample (**Fig. 6**).

52 617 53 54 618 **5. Summary**

56 619 This work has presented results of a time course study of an intracellular biomineralization
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58 620 process elaborating the formation of mature magnetite magnetosomes in cells of the
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621 magnetotactic bacterium *M. magneticum* strain AMB-1. Our results indicate that the
622 initially-produced particles are Fe(III)-rich. Later, some of the Fe(III) is reduced to form
623 magnetite. The particles in the growth phase are primarily disorganized but start forming
624 organized chains at about 4 h of growth and reach a mature stage between 24 to 48 h, with
625 number per cell and size of the magnetosomes similar to that observed in cells at saturation phase
626 grown in Fe-rich culture. Our results are consistent with most previous studies which report the
627 presence of some type of excess Fe(III) during early growth. However, few of these studies
628 resulted in the identification of a specific Fe(III)-rich species. Our Fe L₃ XAS results suggest
629 α -hematite is a precursor to magnetite in AMB-1, but do not exclude other possible precursors
630 such as ferrihydrite. Future studies will reveal whether these two species contribute to the same
631 or to distinct pathways towards magnetite. In addition, this work provides additional insight into
632 the magnetite biomineralization mechanism through detailed Fe L₃ XAS and XMCD spectra of
633 precursors and immature magnetosomes.

634
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5 **787 Figure Captions**

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7 **788 Figure 1** Schematic of XMCD as applied to magnetotactic bacteria. (a) 3D view for tilt = 0°,
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9 **789** with the vector for circular right (CR) polarization along the X-ray propagation direction (black)
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11 **790** and the sample with 3 possible in-plane magnetization vectors (red, blue - horizontal, green -
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13 **791** vertical). (b) Top view for tilt = 0°. There is no XMCD signal since the dot product of the photon
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15 **792** polarization and each of the sample magnetization vectors is 0. (c) 3D view for polar angle tilt of
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17 **793** 30°. (d) Top view for tilt = 30°. There is a 50% XMCD signal with both parallel (red) and
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19 **794** antiparallel (blue) alignment of an in-plane, horizontal sample magnetization. If the sample
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21 **795** magnetization is vertical (green) no XMCD would be observed at any polar tilt angle.
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24 **796**
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26 **797 Figure 2** (a) Images of particles in individual cells from the 9 time course points of batch B. In
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28 **798** each case the image is $OD_{\text{peak}} - OD_{\text{pre}}$ where OD_{peak} is average of 8 images from 707.5 to 711.5
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30 **799** eV, and OD_{pre} is average of 8 images from 702 to 706 eV. Scale bar is 200 nm in each case. (b)
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32 **800** Fe L₃ spectra of particles extracted from stacks measured on the cells in Fig 2a. The zero of each
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34 **801** spectrum is indicated on the vertical axis. The OD/cm scaling is the same for each spectrum. The
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36 **802** arrow indicates the shoulder at 708.4 eV, which is more prominent in the spectra of particles at
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38 **803** the 10 m, 30 m and 2 h time course.
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41 **804**
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43 **805 Figure 3** (a) Stacked bar chart of the numbers and distribution of sizes of the particles per cell
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45 **806** derived from TEM images at each of the 9 time course points (batch B). The protocols used to
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47 **807** achieve an accurate and unbiased distribution are discussed in the text. (b) evolution of numbers
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49 **808** of particles within particle size ranges as a function of log(time). There is a 30 m lag phase
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51 **809** followed by logarithmic growth of particles. Error bars not included for clarity. The
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53 **810** corresponding numerical data, including standard deviation, are shown in Table S-2.
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Figure 4 (a) Average of 36 optical density (OD) images from 704 to 717 eV of an AMB-1 cells from a high Fe content culture of AMB-1 grown over an extended time period. The polarization was circular right (CR) and the sample was tilted 30° relative to the X-ray beam. The numbers at the right side of the image are the OD limits of the grey scale. (b) Color coded composite of component maps derived from a fit of the Fe L₃ stack to spectra for parallel (red), antiparallel (blue) magnetite and the no-Fe signal of the non-magnetite cell contents (green). The arrows indicate the directions of the parallel and antiparallel magnetic orientation. (c) Plot of the average Fe L₃ spectra of all the parallel (red) or anti-parallel (blue) magnetosomes, along with the derived X-ray magnetic circular dichroism (XMCD), as points. The solid lines are the spectra and XMCD reported for synthetic magnetite (Goering *et al.* 2007), scaled to match the optical density. The reference XMCD spectra was divided by 2 since at 30° tilt angle only 0.5 of the magnetic moment is projected on to the photon spin vector.

Figure 5 (a) Fe map of the 8 h sample from batch A from difference of OD images at 709.2 and 700 eV. The rectangles identify precursor (green), intermediate (cyan) and fully formed (red & blue) magnetosomes. (b) XAS spectra recorded with CR of regions identified in (a), plotted on an absolute OD scale without offsets. (c) rescaled and offset plots of the same data, compared to the Fe L₃ spectra of FeCl₃ and the spectra of magnetite recorded with parallel and antiparallel circular polarization (Goering et al 2007). When dots and lines are superimposed (middle chain, precursor chain), the dots are the experimental data while the solid lines are a smoothed version as a ‘guide to the eye’. The other solid lines correspond to unsmoothed experimental data and reference spectra. The good agreement of the spectrum of the big magnetosome with the anti-parallel spectrum of magnetite and that of the left chain with that of the parallel spectrum of magnetite shows that the magnetization vector of the big magnetosome and that of the left chain are oriented in opposite directions. The feature at 708.4 eV is strongest in the precursor chain (green) but is weak or absent in the other two chains, indicating a transition from Fe(III) to pure magnetite.

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Figure 6 Analysis of a cell from 5.7 h sample, batch B. (a,b) TEM images measured after the STXM measurements. (c) Average of all images of the Fe L₃ stack measured with CR with the sample at a tilt angle of 0°. (d) XMCD map (OD_{708.2}-CR - OD_{708.2}-CL) measured with the sample at a tilt angle of 0°. (e) XMCD map (OD_{708.2}-CR - OD_{708.2}-CL) measured with the sample at a tilt angle of 30°. (f) Fe L₃ XAS and XMCD spectra of all the black particles in Fig. 5e, measured with the sample at a tilt angle of 30°.

Figure 7 Fe L₃ spectra extracted from an Fe L₃ stack of the 24 h AMB-1 sample from batch B, measured with CR at a tilt angle of 30°. These five spectra were used to fit the stack, so as to obtain the component maps presented in Figure 8.

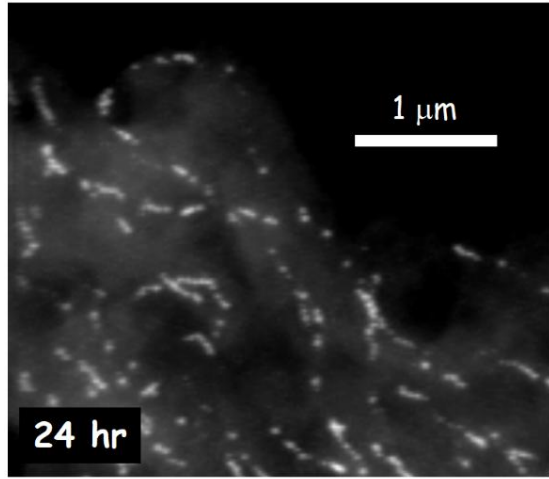
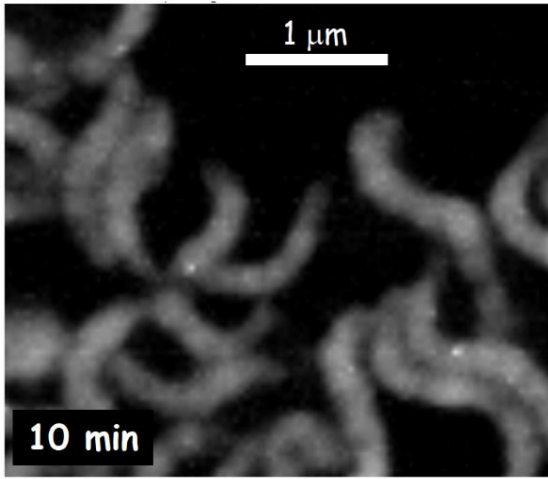
Figure 8 24 h sample from batch B, measured with the sample plate tilted at 30°. (a) image of complete cell at 709.7 eV. (b) average of all 60 images of stack recorded in the top half of the cell. Component maps derived from a 5-component fit of the Fe L₃ image sequence (spectra shown in Fig. 7) of (c) cell, (d) Fe(III), (e) magnetosomes with antiparallel spectra in CR, (f) magnetosomes with parallel spectra in CR, (g) ‘rim’ signal, showing a band of Fe-containing material along the outer boundaries of the expanded part of the cell (bright in Fig. 8c). This spectral component also maps signals in the magnetosome chain. See Fig. S-5 for results of an alternate fit. (h) color coded composite of maps of the antiparallel (red), cell (green) and parallel (blue) component maps.

Figure 9 Fe L₃ spectra of the magnetosomes in the 24 h AMB-1 cell of batch B recorded with CR and CL with the sample tilted at 30°. The resulting XMCD signal is also plotted. In each case the data from the magnetosomes of the cell from the 24 h sample is overplot with the corresponding spectra from pure magnetite (Goering et al, 2007).

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865 **Graphical Abstract**

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Figure 01
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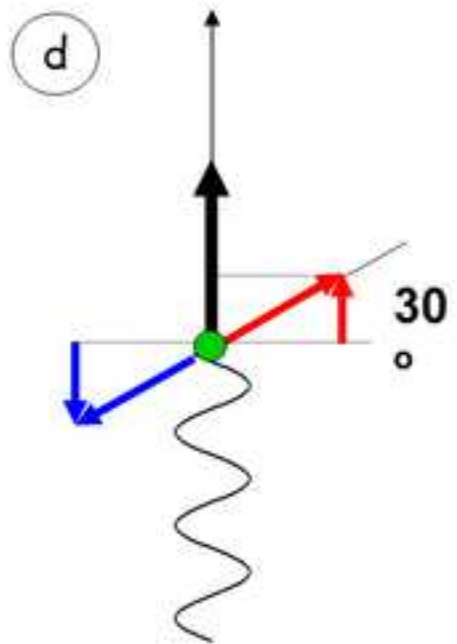
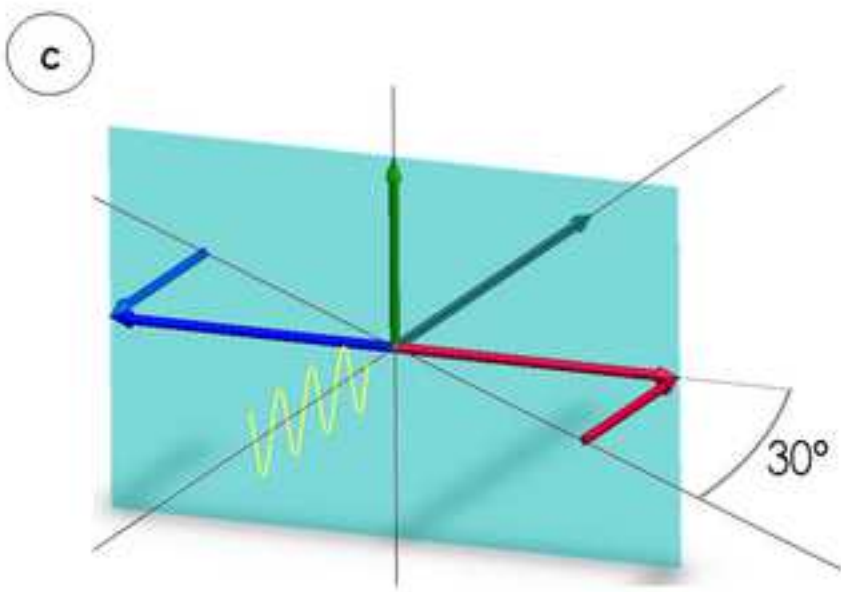
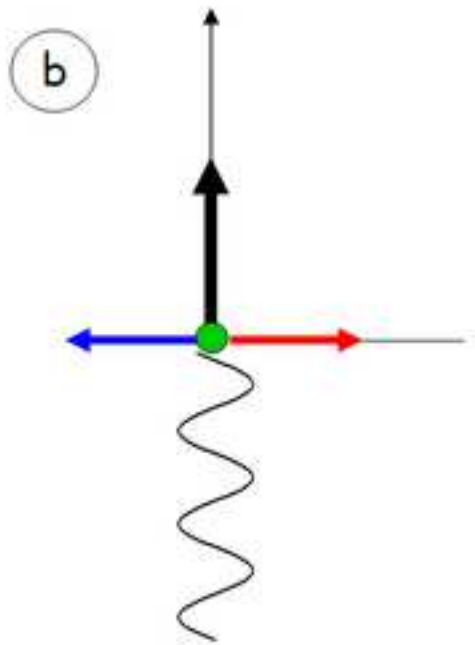
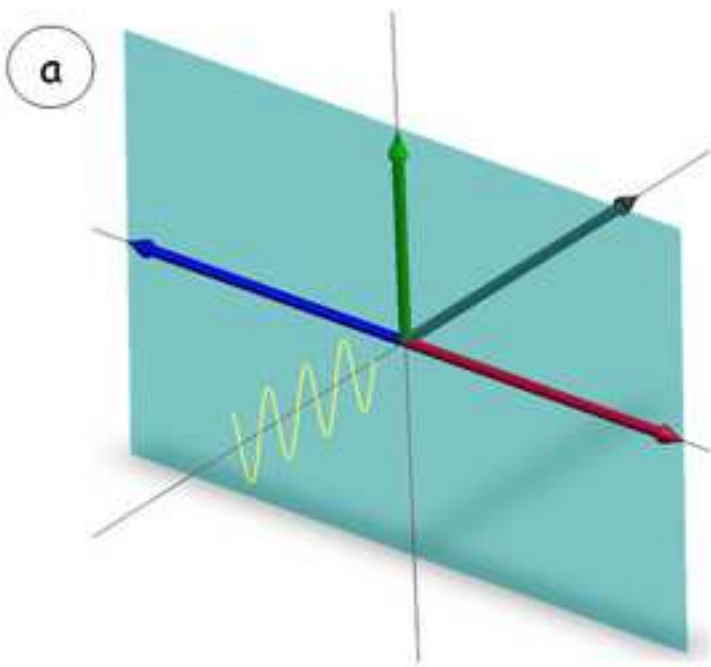
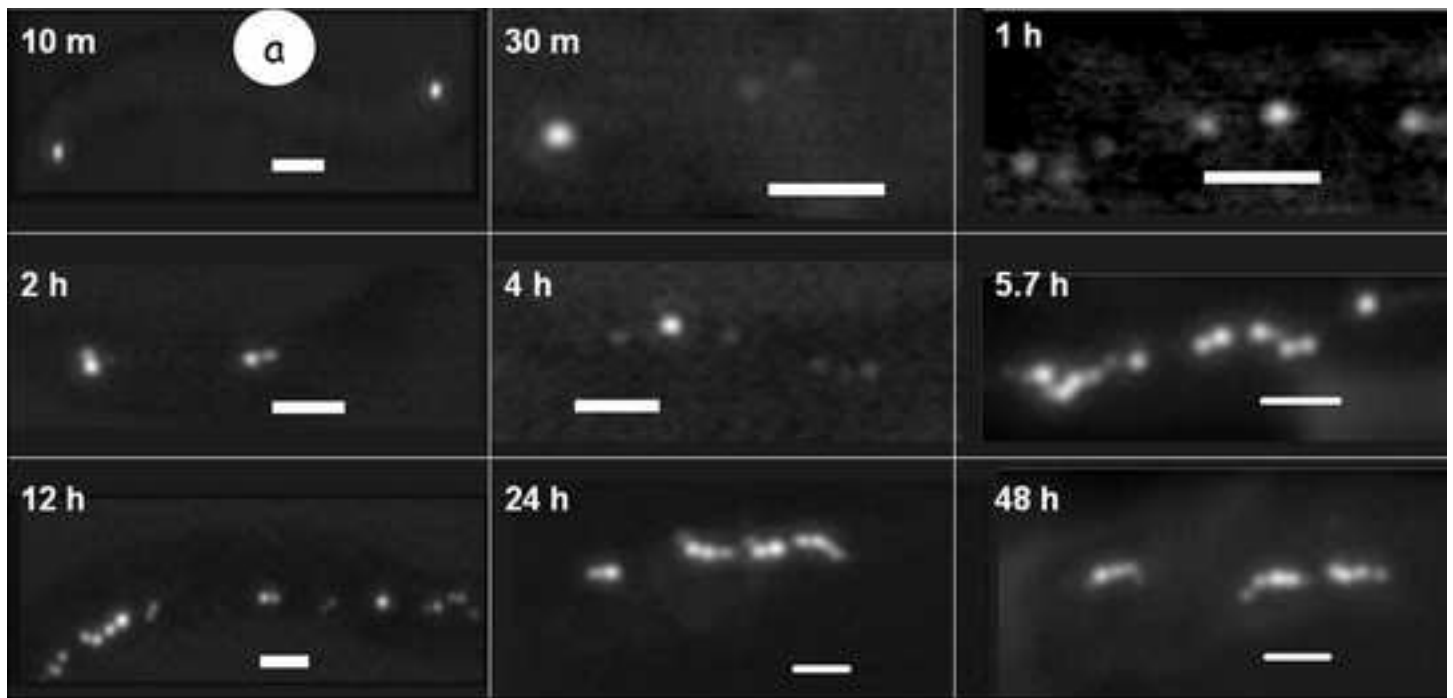


Figure 02
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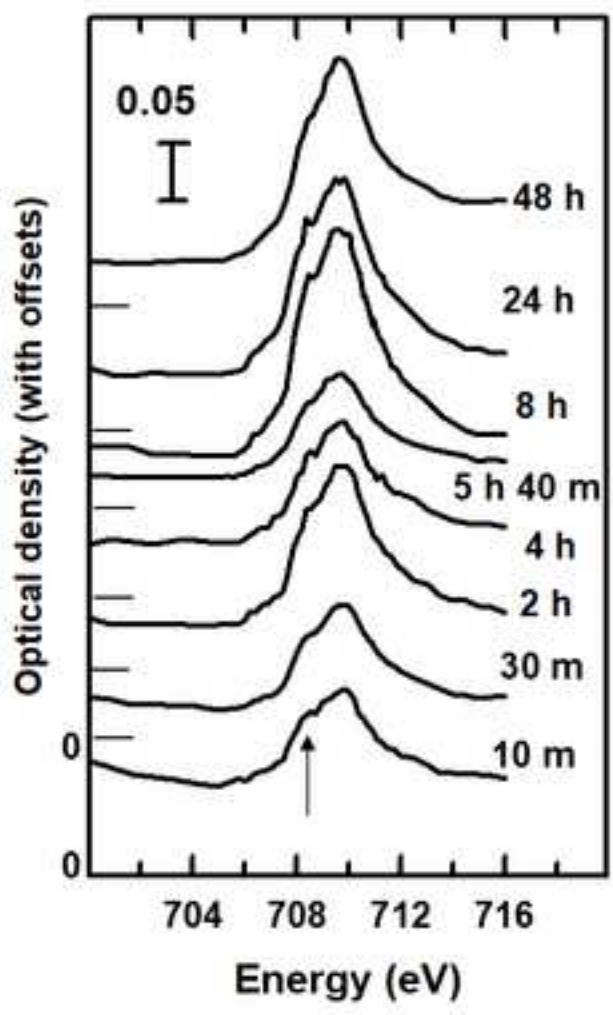


Figure 03

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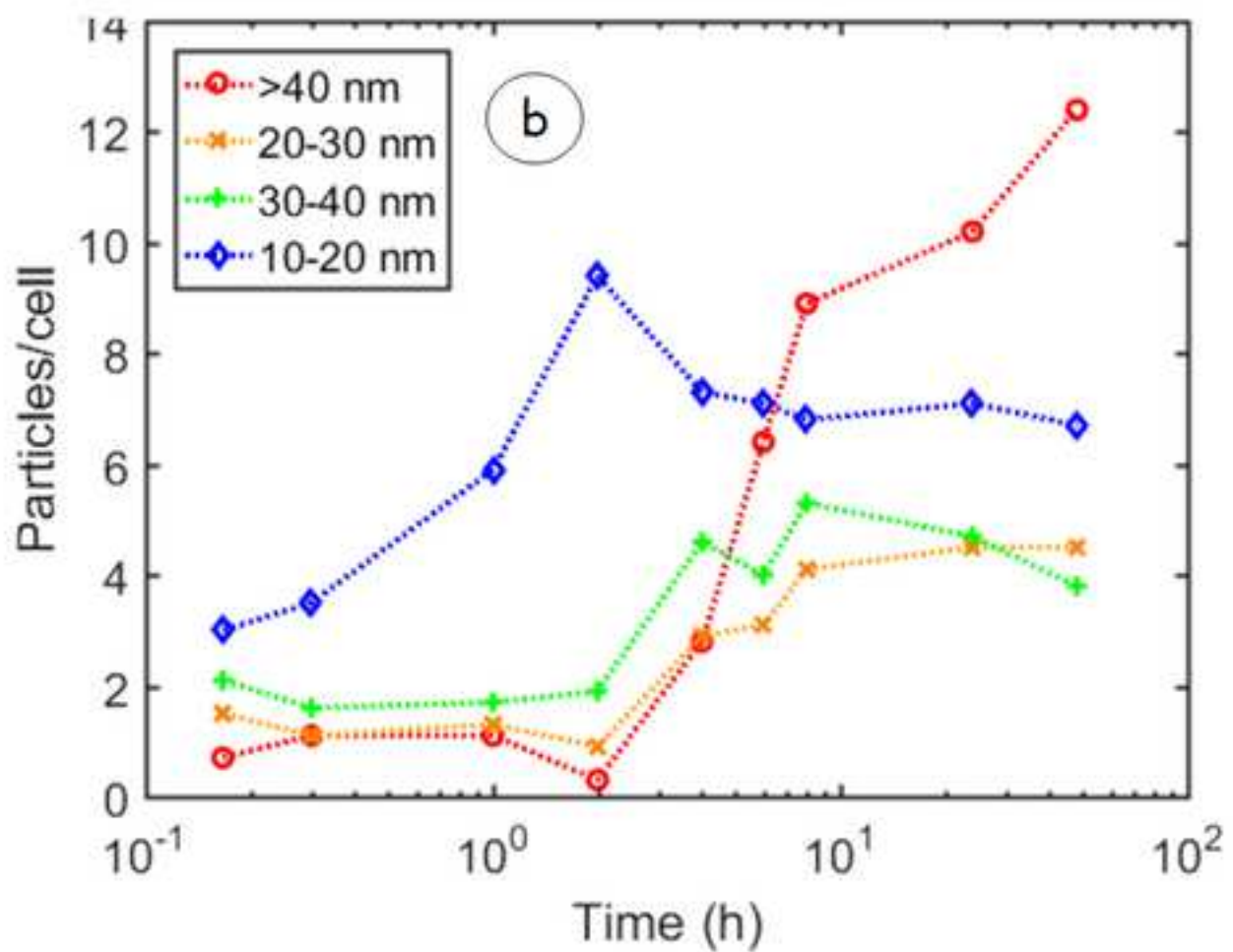
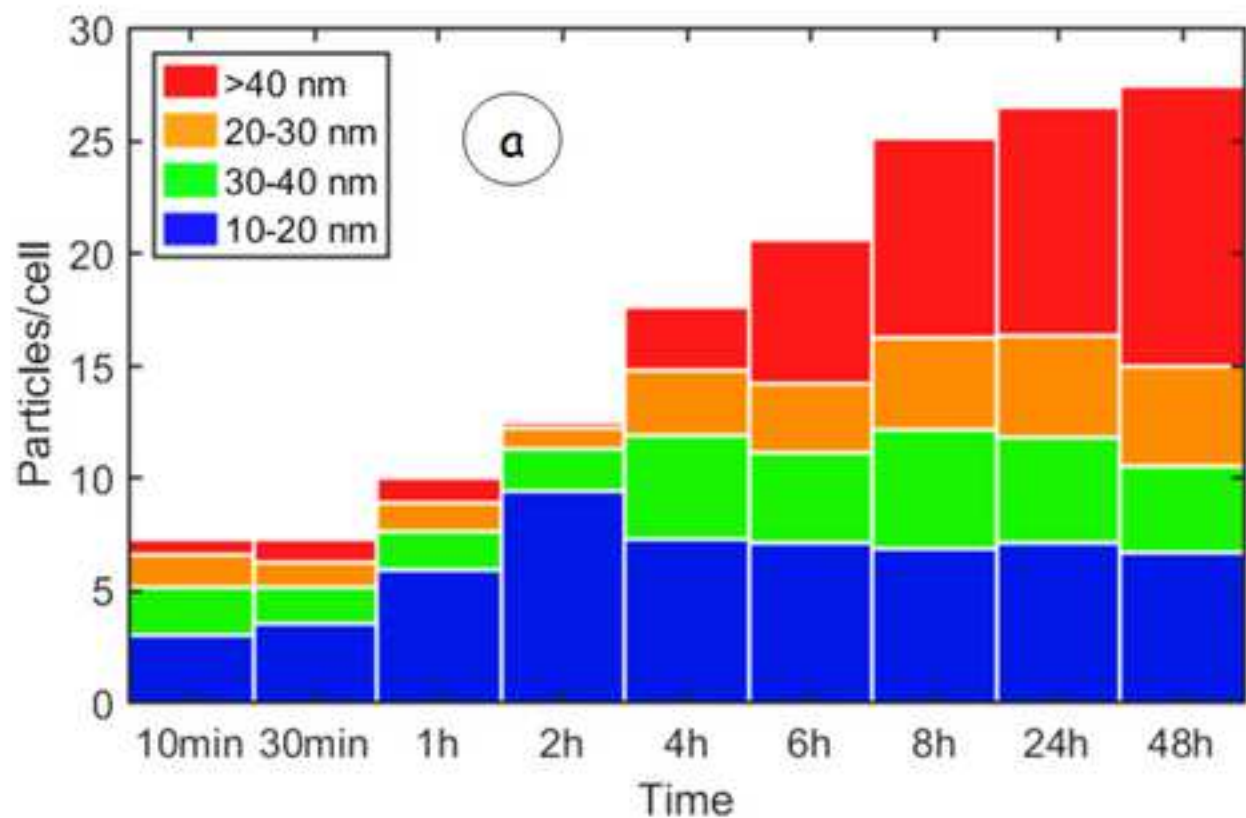


Figure 04

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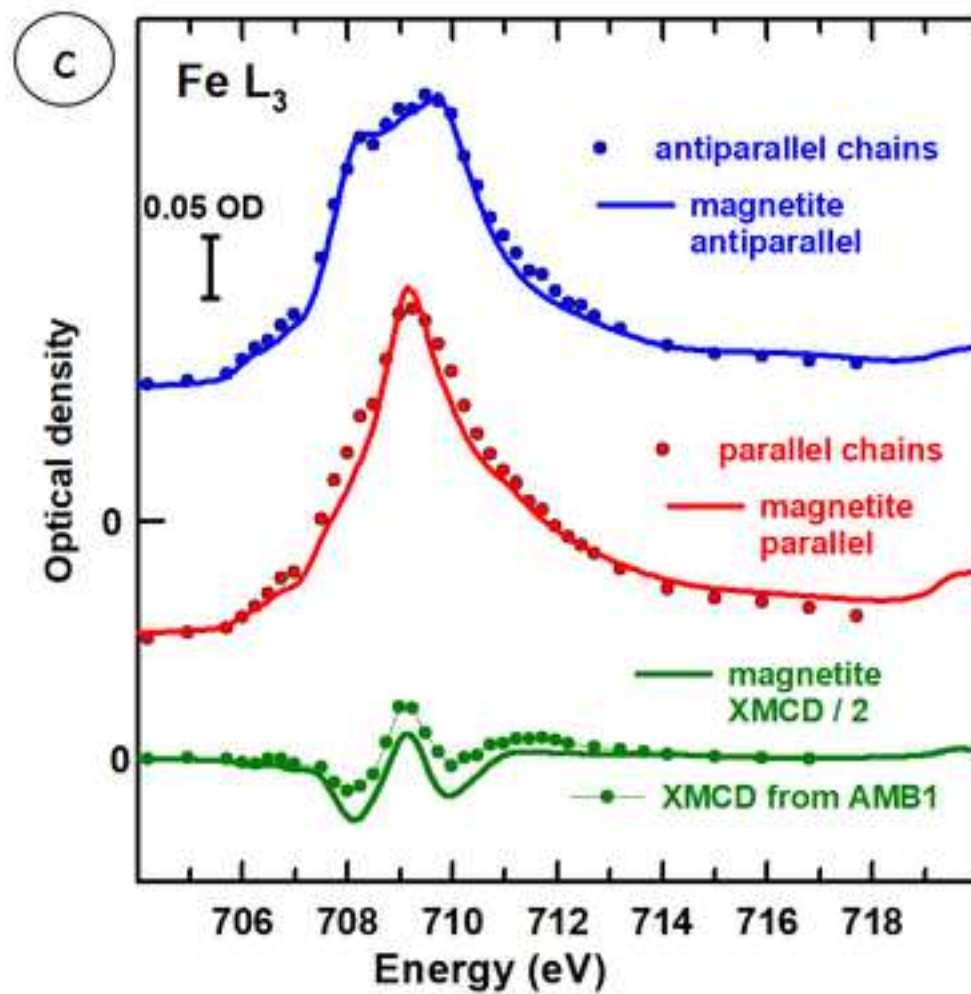
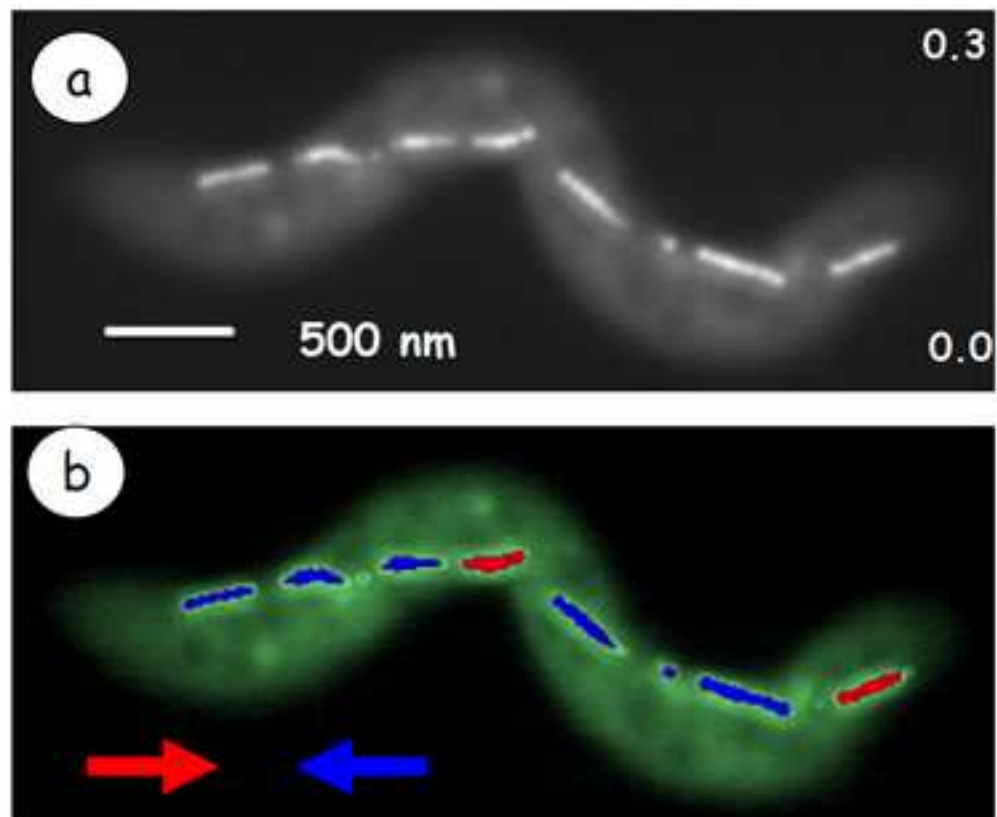


Figure 05

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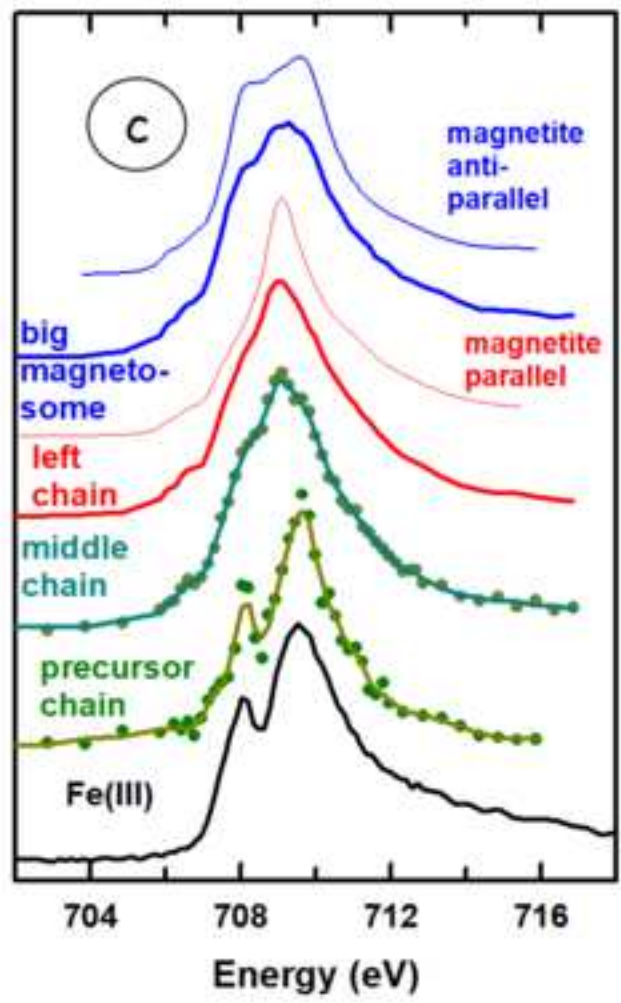
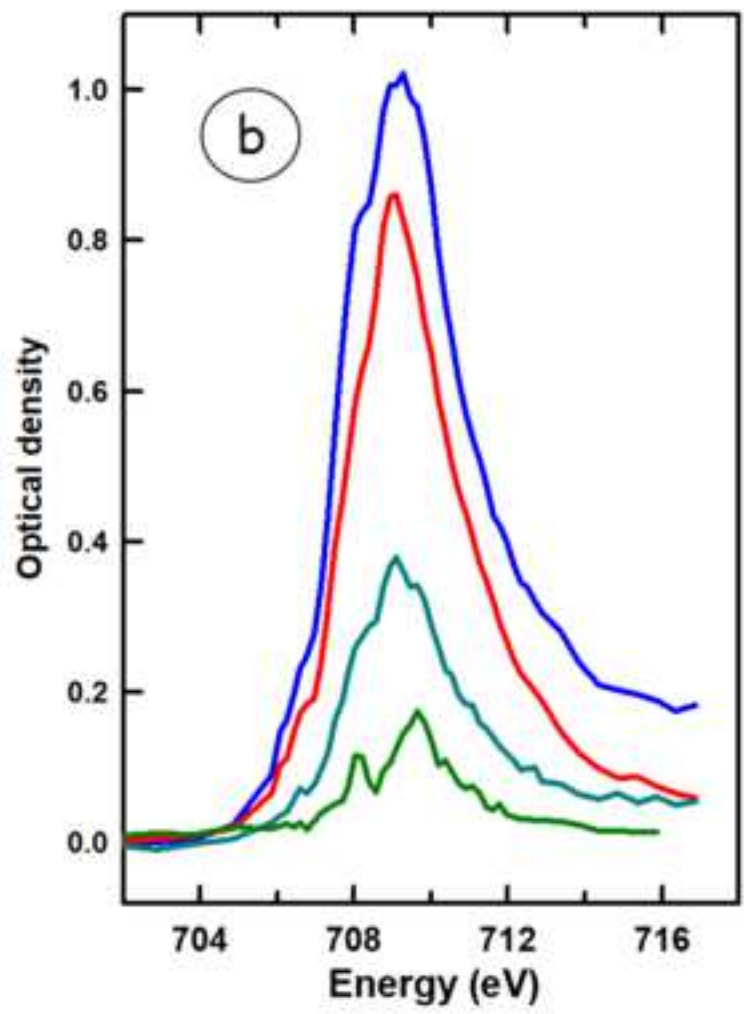
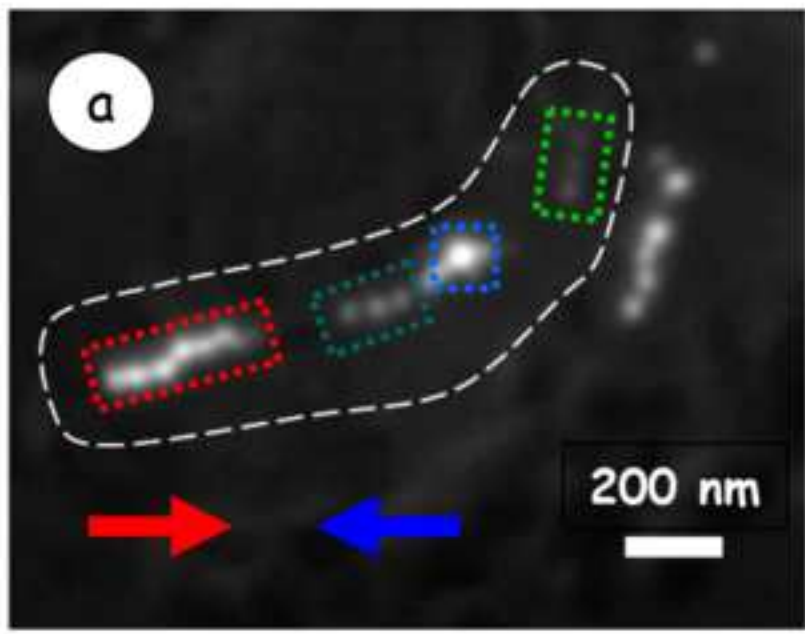


Figure 06

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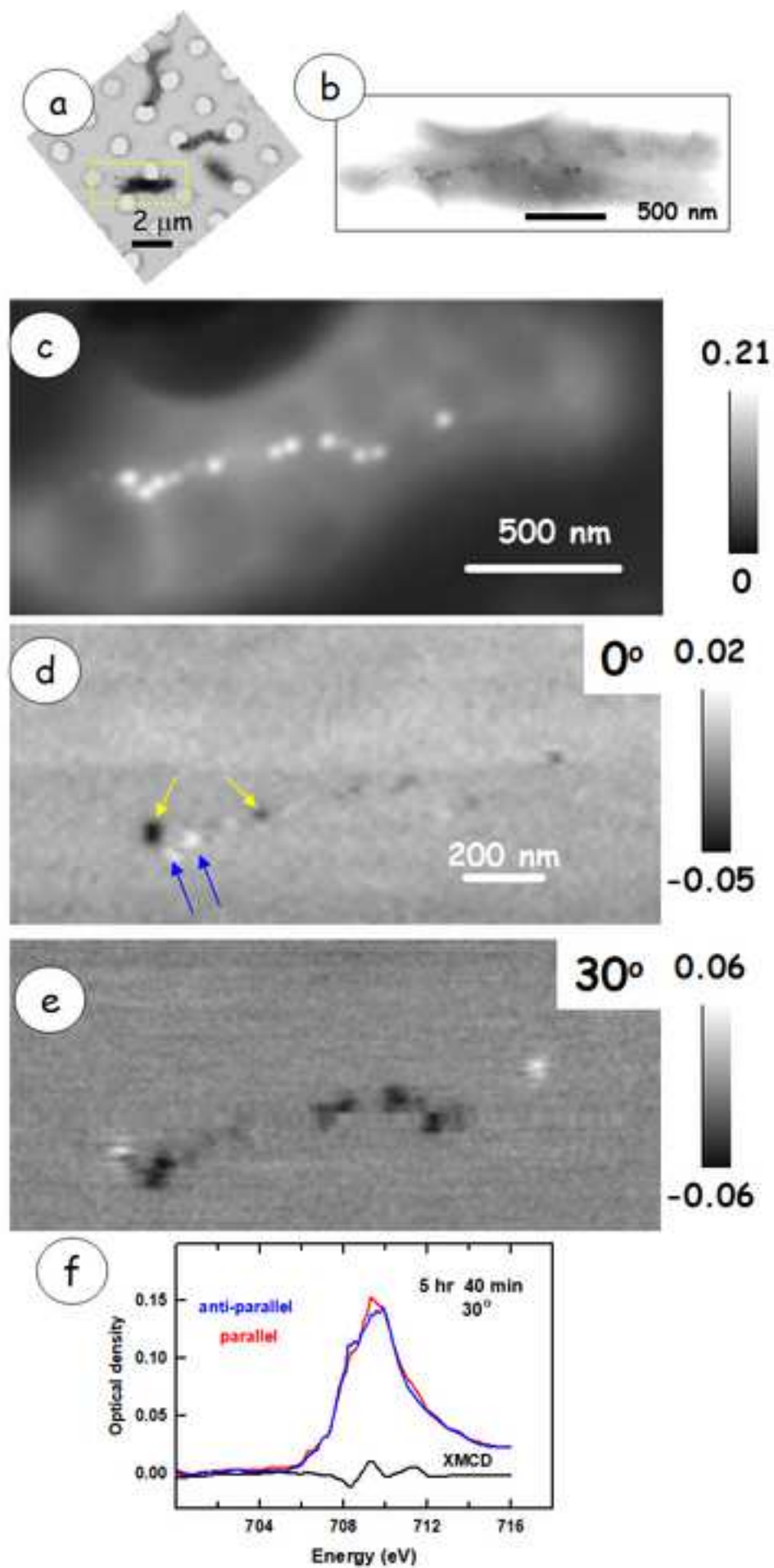


Figure 07
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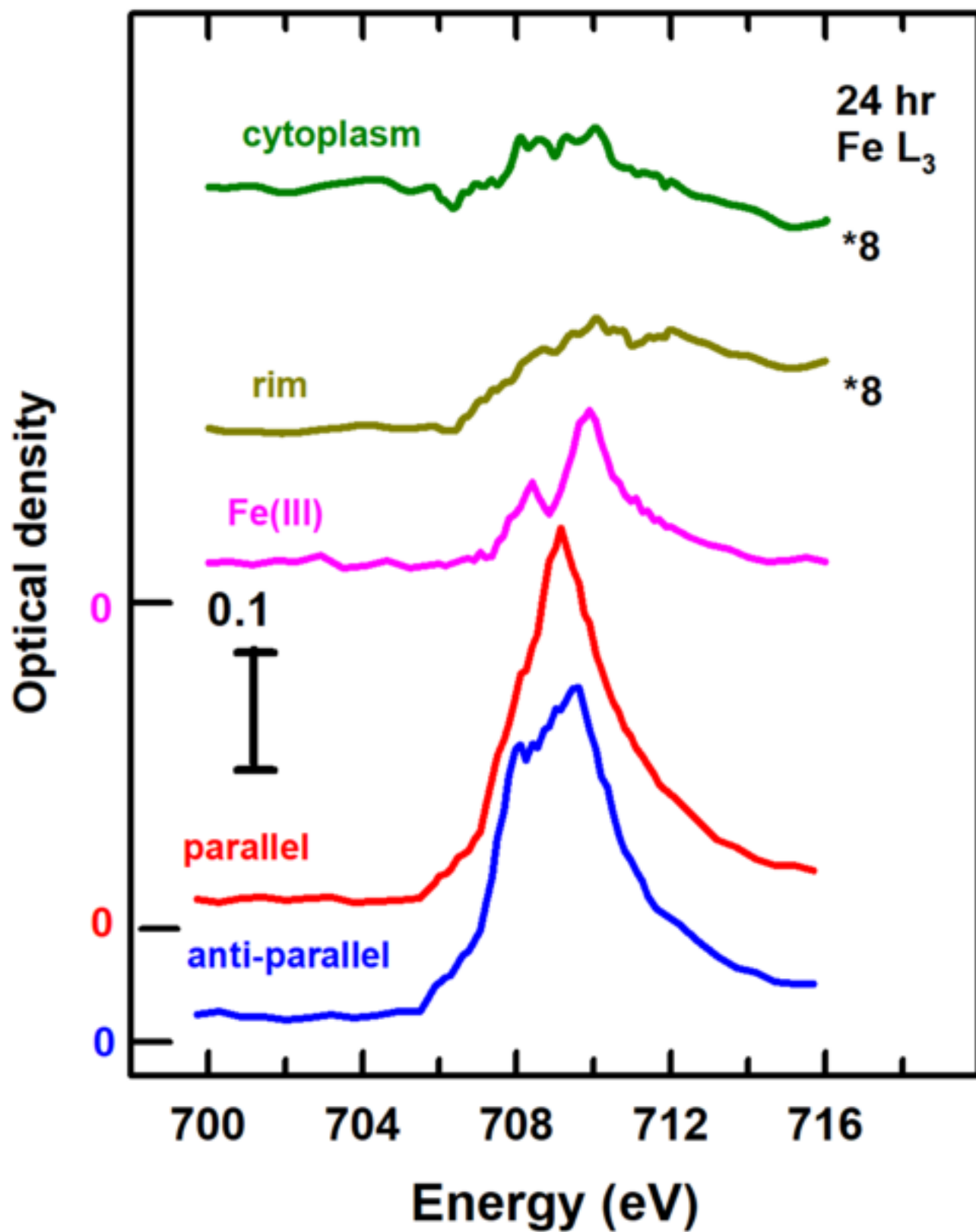


Figure 08

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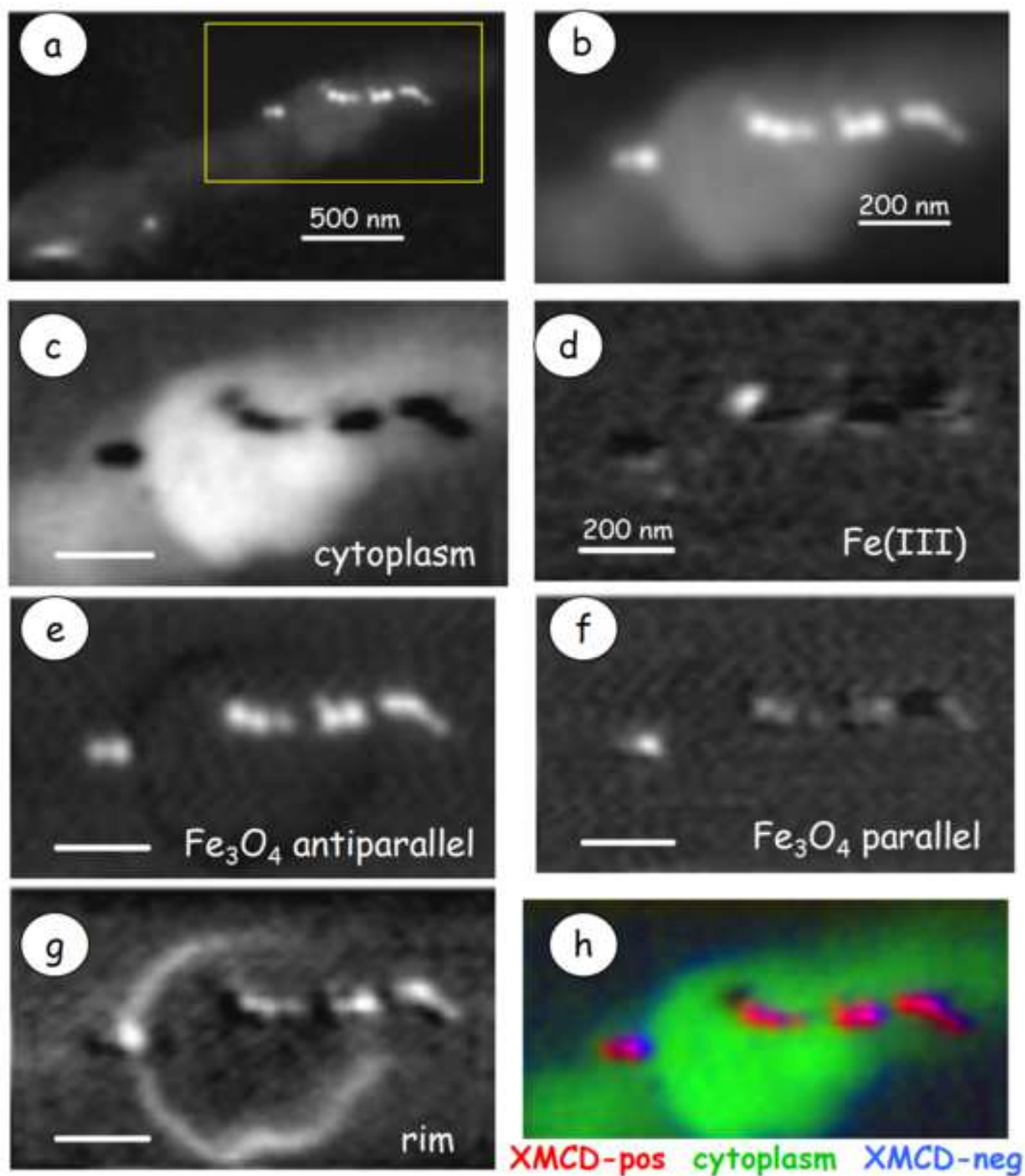
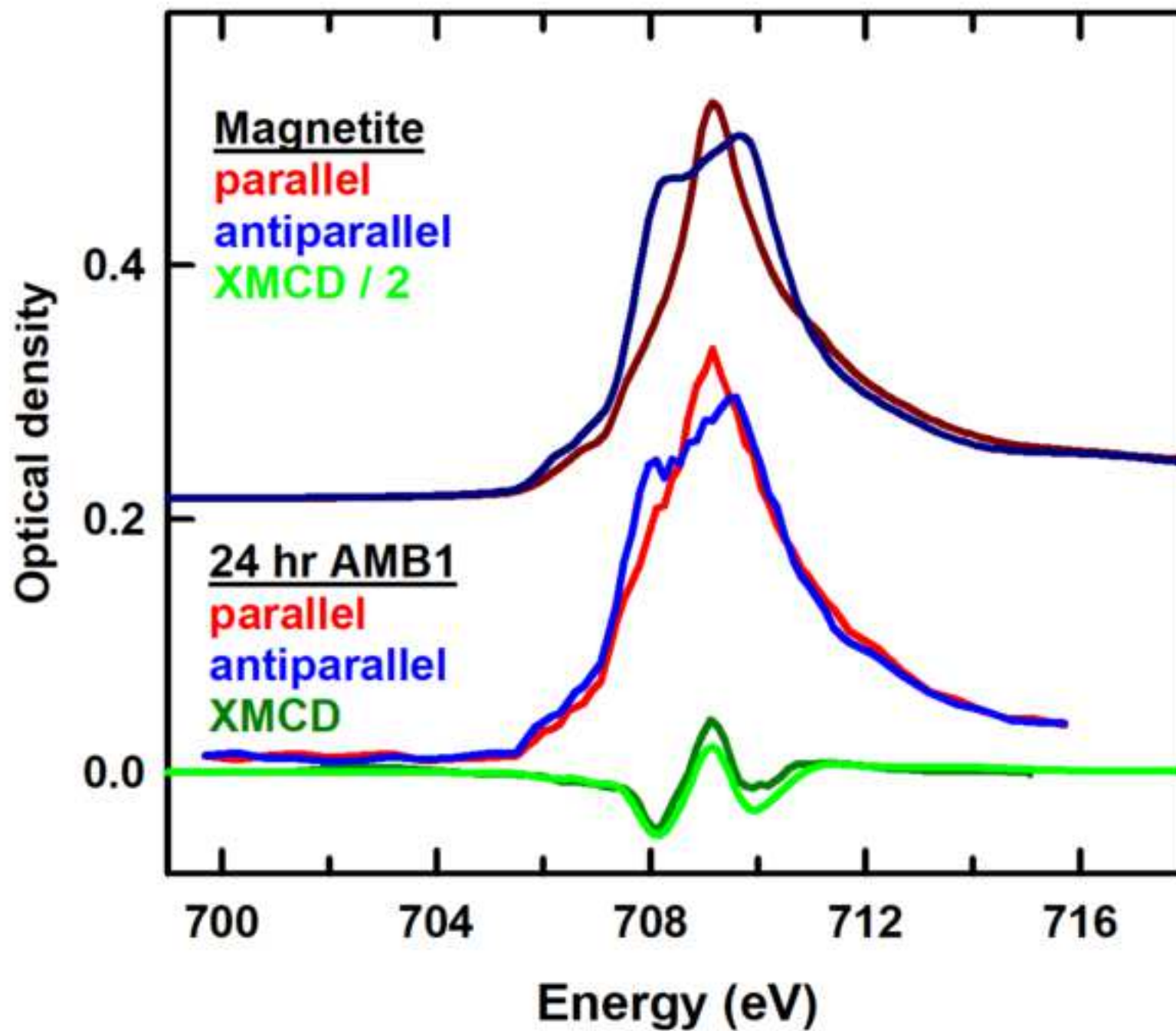


Figure 09
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Supplementary information file

[Click here to download Background dataset for online publication only: AMB1-time-course-STXM-SI-revised-submitted.pdf](#)