Nanoscale analysis of pyritized microfossils reveals differential heterotrophic consumption in the ~1.9-Ga Gunflint chert

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The 1.88-Ga Gunflint biota is one of the most famous Precambrian microfossil lagerstätten and provides a key record of the biosphere at a time of changing oceanic redox structure and chemistry. Here, we report on pyritized replicas of the iconic autotrophic *Gunflintia*–*Huroniospora* microfossil assemblage from the Schreiber Locality, Canada, that help capture a view through multiple trophic levels in a Paleoproterozoic ecosystem. Nanoscale analysis of pyritic *Gunflintia* (sheaths) and *Huroniospora* (cysts) reveals differing relic carbon and nitrogen distributions caused by contrasting spectra of decay and pyritization between taxa, reflecting in part their primary organic compositions. In situ sulfur isotope measurements from individual microfossils ($\delta^{34}\text{S}_{\text{V-CDT}} +6.7\text{‰}$ to $+21.5\text{‰}$) show that pyritization was mediated by sulfate-reducing microbes within sediment pore waters whose sulfate ion concentrations rapidly became depleted, owing to occlusion of pore space by coeval silicification. Three-dimensional nanotomography reveals additional pyritized biomaterial, including hollow, cellular epibionts and extracellular polymeric substances, showing a preference for attachment to *Gunflintia* over *Huroniospora* and interpreted as components of a saprophytic heterotrophic, decomposing community. This work also extends the record of remarkable biological preservation in pyrite back to the Paleoproterozoic and provides criteria to assess the authenticity of even older pyritized microstructures that may represent some of the earliest evidence for life on our planet.

biogeochemistry | taphonomy | paleontology

Pervasive pyritization of soft-bodied organisms is rare but may result in remarkable cellular preservation and provide unique biogeochemical and taphonomic information (1–5). Pyritic microfossils have been reported from several Precambrian strata (e.g., ref. 6), with the oldest examples cited as some of the earliest evidence for life on our planet (7). These hold great potential for better understanding Precambrian biology and environmental conditions, but few data have been retrieved from them beyond simple morphological descriptions, because their opacity makes them very difficult to examine using conventional microscopic methods. Indeed, the biogenicity of many Precambrian pyritic microfossils may be questioned (8) owing to their apparent occurrence as simple, solid filaments and spheres; the lack of preserved chemical and/or isotopic biosignatures; and a poor understanding of how these pyritic objects relate taxonomically to bona fide Precambrian carbonaceous microfossils.

The 1.88-Ga Gunflint Formation occupies a key point in Earth's history. It shortly predates the earliest widely accepted evidence for fossil eukaryotes (9) and the generally accepted timing of the transition from largely ferruginous to largely sulfidic ocean conditions (10, 11). Recent work suggests that this

transition was prolonged and spatially variable, with oxygenated surface waters potentially underlain by sulfidic wedges and deeper ferruginous waters for much of the mid- to late Proterozoic (12, 13). Hence, pyritic microfossils are of interest for information they may reveal about the geochemical cycles of iron and sulfur, as well as carbon, at this time.

Although pyrite is relatively common within the Gunflint Formation (14, 15), pyritized microfossils are localized (13, 14) and, hitherto, have not been analyzed in detail. Previous work focused upon carbonaceous microfossils, especially those from shallow-water, near-shore chert facies (14–20). The dominant components of this biota are segmented filaments and enclosing tubular sheaths (*Gunflintia* spp.) plus rounded, coccoid vesicles (*Huroniospora* spp.), interpreted as photoautotrophs (15, 17, 20, 21). Microbial iron oxidation also has been invoked for hematite-encrusted filaments and for rare rods and coccoids within subtidal chert facies (22, 23). Below, we expand our understanding of the Gunflint biota by documenting evidence of biological trophic levels and taphonomic pathways within the shallow-water stromatolitic chert facies of the type locality (14) at Schreiber Channel, Canada.

Pyritic microfossils occur abundantly in thin sections from Schreiber Channel. Assemblages are dominated by the empty sheaths of *Gunflintia* (~90%) together with simple, well-rounded hollow vesicles of *Huroniospora* sp. (~9%) and rare *Gunflintia* trichomes (see Fig. S1 and SI Discussion for Gunflint taxonomy). Pyritized assemblages pass laterally into laminar zones containing carbonaceous *Gunflintia* and *Huroniospora* (Fig. 1A), although some individual *Gunflintia* sheaths may be seen changing from carbonaceous to pyritic along their length (Fig. 1B). Most pyritic microfossils, including those with the highest quality of preservation, comprise replicas that sit within submillimetric patches of entirely pyritized organic material, surrounded by small zones of clear chert (Fig. 1A and Fig. S2A). More rarely, pyritic microfossils occur in direct contact with carbonaceous microfossils (Fig. 1B) or as extensive pyritized microbial mats (Fig. S2B), in which microfossil morphology is poorly preserved.

The two main taxa show very distinctive patterns of preservation. Like their carbonaceous precursors, pyritized *Huroniospora* vesicles (Fig. 2A) are hollow and range in diameter from ~3–15 μm

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Fig. 1. Occurrence of pyritic microfossils at Schreiber Channel. (A) Stromatolitic chert with microfossil-rich laminae. Pyritic microfossils occur most commonly in millimeter-sized patches surrounded by clear chert (circled). These patches frequently pass laterally into areas rich in organic material and carbonaceous microfossils. (B) Laser Raman map (*Inset*) showing filamentous sheaths of *Gunflintia* that are part carbon (red) and part pyrite (green).

(mean, 8.2 μm ; $n = 62$). Their pyritized walls often exceed 1 μm (mean, 1.1 μm ; $n = 35$), which represents a significant increase in microfossil wall thickness compared with co-occurring carbonaceous examples [maximum, 600 nm (24)], and they also show a moderate increase in microfossil diameter [carbonaceous examples, 3–10 μm in diameter (mean, 6.8 μm ; $n = 52$)]. The walls comprise microcrystalline pyrite grains (~ 1 –2 μm in size) whose crystallographic orientations change little across the microfossil and enclose nanograins of silica (Fig. S3). This contrasts with carbonaceous examples in which the walls have a sawtooth-like ridged texture (24), comprising largely continuous rings of carbon disrupted by nanograins of silica (Fig. 2B).

Changes in morphology and wall structure also are seen in *Gunflintia* sheaths (Fig. 2C and D). Although both carbonaceous and pyritized *Gunflintia* have similar mean filament diameters of 1.8 μm , the walls are much thicker in the pyritized examples, comprising up to 90% of the total fossil diameter (mean, 59%; $n = 84$), but their hollow nature remains evident (Fig. 2C). The pyritized walls also contain nanograins of silica, although not as numerous as in *Huroniospora*. In all cases studied, the walls of carbonaceous *Gunflintia* display conspicuous holes (Fig. 2D).

Additional pyritized material occurs close to well-preserved pyritic specimens of *Huroniospora* and *Gunflintia*. Especially notable are very small hollow ellipsoids and spheroids of rather

Fig. 2. Changes in microfossil morphology and ultrastructure during pyritization. (A) Pyritized *Huroniospora* (bright-field TEM image) demonstrating thick (up to ~ 2 μm) pyrite walls (dark gray) enclosing numerous nanograins of silica (pale gray; arrow). (B) Carbonaceous *Huroniospora* (bright-field TEM image) with thinner walls (mostly ~ 200 nm) comprising a ring of carbon (white/pale gray) with a sawtooth texture caused by impinging silica nanograins. (C) Pyritized *Gunflintia* sheath (energy-filtered TEM image showing iron distribution) demonstrating thick pyrite walls (~ 500 nm) and pyrite overgrowths. (D) Carbonaceous *Gunflintia* sheath (energy-filtered TEM image showing carbon distribution) showing poorer quality of preservation than *Huroniospora*, with walls comprising discontinuous rings of carbon. Nanograins of silica once again impinge upon and may be included within these walls (arrow). B and D were modified from (24), Copyright (2012) with permission from Elsevier.

uniform size (800 nm to 1.2 μm) and most commonly found attached to or partially embedded within *Gunflintia* filaments (Fig. 3 and Fig. S4). These epibionts have mean diameters an order of magnitude smaller than *Huroniospora* spheres and only rarely are attached to *Huroniospora*. The remainder of the pyritic material occurs as “irregular masses,” often co-occurring with the epibionts (Fig. 3B and Fig. S4).

Nanoscale chemical mapping using NanoSIMS reveals distinctive patterns of carbon and nitrogen within the pyritized microfossils (Fig. 4). All pyritized components retain at least small amounts of carbon and nitrogen consistent with remains of an organic precursor. However, the patterns of nitrogen enrichment differ markedly between taxa. *Gunflintia* typically have both carbon and nitrogen scattered in low levels throughout the entire pyritized microstructure, plus some randomly situated hotspots of nitrogen (Fig. 4A). A similar pattern of dispersion and hotspots may be seen within the irregular masses of pyrite, whereas the pyritized epibionts attached to *Gunflintia* frequently contain relatively high levels of nitrogen (Fig. 4B). In contrast, pyritized *Huroniospora* typically display a narrower, ring-shaped distribution of nitrogen (Fig. 4C), closely resembling the morphology of the microfossil wall in co-occurring carbonaceous specimens (compare Figs. 4C and 2B).

In situ sulfur isotope data (Tables S1–S3) were collected both from individual pyritized microfossils (Figs. S5 and S6) and from clusters of microfossils. The total range in $\delta^{34}\text{S}_{\text{V-CDT}}$ was +6.7‰ to +21.5‰ (mean, +14.1‰; $n = 41$). Data from two different instruments show similarity in both range and mean values (Cameca IMS 1280: +6.9‰ to +21.5‰; mean, +16‰; $n = 21$; NanoSIMS 50: +6.7‰ to +20.5‰; mean, +12‰; $n = 20$). No taxon-specific patterns were preserved. In situ sulfur isotope data also were obtained from micrometer-sized pyrite cubes not associated with microfossils and lacking carbon and nitrogen. These data were similar to those obtained from the microfossils with a $\delta^{34}\text{S}_{\text{V-CDT}}$ range from +7.2‰ to +22.2‰ (mean, +14.0‰; $n = 15$).

The isotopic data reveal the mechanism of microfossil pyritization. During microbial sulfate reduction (MSR), the lighter ^{32}S isotope is reduced more rapidly than ^{34}S (25). Hence, dissolved sulfide becomes enriched in ^{32}S , and this is incorporated into pyrite. If the system remains open to the sulfate source, then the resultant pyrite will have a narrow range of light $\delta^{34}\text{S}$ values.

However, if the pore waters become isolated from overlying seawater, the residual pore water sulfate becomes progressively enriched in ^{34}S and produces heavier $\delta^{34}\text{S}$ in the resultant pyrite. Our pyrite is isotopically heavy, showing a maximum $\delta^{34}\text{S}_{\text{sulfate-pyrite}}$ fractionation of about 14‰, with the heaviest $\delta^{34}\text{S}$ values approximating the $\delta^{34}\text{S}$ of the Paleoproterozoic seawater sulfate ($\delta^{34}\text{S}_{\text{V-CDT}} = +20 \pm 2\text{‰}$) (26). These patterns may be explained best by MSR in anoxic microenvironments within sediment pore waters that initially were open to seawater containing moderate concentrations of sulfate (27) but that quickly evolved to a state of sulfate limitation because of occlusion of pore space by contemporaneous silicification. Although hydrothermal fluids might pyritize filaments abiogenically, this likely would result in less-positive $\delta^{34}\text{S}$ values (28) plus a more homogenous signature over this spatial scale.

Our isotopic data therefore provide clear evidence that heterotrophic metabolic pathways were present in the *Gunflint* microbiota. We go further, with evidence that some preserved microfossils were heterotrophic, consuming preformed organic matter. Reflected light mapping and 3D nanotomographic reconstructions (Fig. 3 and Fig. S4) reveal multiple ellipsoidal to spheroidal epibionts externally attached to or partially embedded within *Gunflintia*. This distribution of epibionts, together with their uniform size, ellipsoidal shapes, hollow interiors, and high levels of residual carbon and nitrogen (Fig. 4B) all are consistent with saprophytic heterotrophs actively decomposing *Gunflintia* sheaths just before pyritization. One alternative possibility that deserves consideration is that the epibionts were merely inorganic blebs of carbonaceous matter whose centers became degraded, with exteriors coated with a rim of pyrite (29). Such a possibility, however, does not explain our evidence for their mainly (bacteria-like) elliptical shape, rather uniform diameter, and preferred association with a single host taxon. Further supporting evidence for our saprophytic interpretation comes from the dispersed nature of carbon and nitrogen throughout the pyritic *Gunflintia* walls (Fig. 4A) and significant increases in pyritized wall thickness compared with carbonaceous precursors, suggesting that organic sheath material was broken up, dispersed, and partially consumed by the saprophytic heterotrophs.

It also might be that these structures were symbiotic epibionts, either using metabolic products or contributing them to the host (cf. ref. 30). Inferences about symbiotic associations, however,

Fig. 3. Morphological evidence for saprophytic heterotrophs and EPSs. (A) Reflected light images of ellipsoidal-spheroidal epibionts up to $\sim 1\text{ }\mu\text{m}$ in diameter (arrows) attached to or embedded within *Gunflintia* and, more rarely, *Huroniospora* (H). (B) Three-dimensional reconstruction and visualization of the pyritic *Gunflint* biota (reconstructed from ~ 80 individual FIB-SEM images spaced 75 nm apart). Micrometer-sized pyritic spheres/ellipses (orange) are attached to or embedded within pyritized *Gunflintia* sheaths and are interpreted here as prokaryotic, saprophytic heterotrophs. Other pyritic material (yellow) is attached or occurs close to *Gunflintia* sheaths; this has neither a crystalline nor cellular morphology and is interpreted here as pyritized EPSs. (Inset) Single FIB-SEM slice indicating the hollow nature of the pyritic *Gunflintia* sheaths (G) and saprophytic heterotroph (arrow), as well as inferred pyritized EPSs.

Fig. 4. Chemical biosignals preserved within pyritic microfossils. (A and B) NanoSIMS ion images of sulfur ($^{32}\text{S}^-$) and nitrogen ($^{26}\text{CN}^-$) from three pyritic *Gunflintia* microfossils (1–3). The pyrite frequently contains a chemical biosignal in the form of nitrogen enrichments. These are spatially variable both within an individual microfossil (1) and between microfossils (1–3). Subcircular hotspots of nitrogen (dashed circle in B) frequently correlate with microspheroids observed in reflected light images, interpreted here as saprophytic heterotrophs. (C) Three-color overlay of NanoSIMS ion images from a pyritic *Huroniospora* microfossil: blue, pyrite; red, oxygen; and green, nitrogen. Note the discontinuous ring of nitrogen (green/yellow) within the pyritized microfossil, which is interpreted to represent a chemical ghost of the original organic microfossil wall. Hotspots of nitrogen exterior to the original wall (arrow) may represent mobilized organics from the original *Huroniospora* wall, the remains of saprophytic heterotrophs, or EPSs. Pores within the pyritic wall have been filled by nanograins of silica (red).

ideally would require evidence for attachment of the epibionts to the surfaces of living *Gunflintia* cells, not just to their presumably abandoned sheaths. Such evidence has not yet been discovered.

The irregular masses of pyrite, which also contain carbon and nitrogen biosignals, are interpreted as the fossilized remains of extracellular polymeric substances (EPSs). EPSs might have been secreted by the primary autotrophic community or by the decomposing heterotrophic community (31); we favor the latter owing to the close association of EPSs with epibionts and decaying *Gunflintia*.

The nanoscale elemental patterns observed in pyritized *Huroniospora* suggest less decay of precursor organic material in this taxon. Here, biochemical remnants of original organic walls appear to be retained in the form of narrow rings of nitrogen enrichment (Fig. 4C). This hypothesis is consistent with observations of fewer heterotrophic epibionts and EPSs attached to *Huroniospora*. However, *Huroniospora* did not completely avoid decay by heterotrophs during pyritization, as evidenced by the residual nitrogen rings being less continuous than in nearby carbonaceous *Huroniospora* walls (Fig. 2B). Thus, the hollow tubes of *Gunflintia* may be interpreted best as the remains of a polysaccharide sheath of moderately refractory composition, in which the most labile components of the cytoplasm and cell membrane were not preserved (Fig. 5); and *Huroniospora* preserves the remains of a cyst with specially thickened walls (18), of a more markedly refractory composition, typically lacking the remains of any cell membrane within.

Preservation of coexisting, carbonaceous, and pyritized microfossil taxa by early silicification means we can document ecosystem components that have been lost, those that have been retained, and those that have been gained in this taphonomic window (Fig. 5). Most of the more labile components of the photoautotrophic assemblage were lost before silicification, including the interior cytoplasm and cell membranes of the vegetative cells. Exceptions to this include cell membranes preserved within relatively rare *Gunflintia* trichomes (14, 15) and occasional putative inner bodies within *Huroniospora* (24). Perforations in the walls of carbonaceous examples of *Gunflintia* sheaths (Fig. 2D) suggest that aerobic heterotrophic and/or physicochemical

degradation affected the sheath. This was followed by more intense anaerobic decay by heterotrophs, including sulfate-reducing bacteria, which led to pyritization. It is possible that cell surface fixation of iron by *Gunflintia* inhibited its autolytic enzymes on death (32), leaving the heterotrophs a significant volume of

Fig. 5. Typical trajectories of differential fossil decomposition and pyritization within the two dominant elements of the Gunflint microbiota seen at the Schreiber locality: prokaryotic sheaths of *Gunflintia* sp. (G) and prokaryotic cysts of *Huroniospora* sp. (H). Early diagenetic silicification has arrested microfossil decomposition at various stages (A–E). (A) Sheaths and cysts still contain cell membranes with cytoplasmic contents (light green). (B) Cell cytoplasm plasmolyzed or decomposed, whereas the cell membrane remains relatively intact (green and blue inner rings). (C) The cell membrane decomposed, leaving only the sheath (G) and the cyst (H). (D) Aerobic heterotrophs (orange) break up the more labile sheath (G) but not the more refractory cyst (H). (E) Microbial sulfate reduction by anaerobic heterotrophic prokaryotes (brown) brings about pyritic replacement (gray-black) of both sheath (G) and cyst (H) material, involving dilation of carbonaceous and nitrogenous matter in the more labile sheath (G) or by marginal addition in the more refractory cyst (H).

material on which to feed. Although the sheath wall structure was lost, the overall tubular morphology was retained and augmented by the growth of pyrite crystals, as well as pyritized heterotrophs and EPSs. In comparison, *Huroniospora* cysts retain more evidence of their original wall structure in both carbonaceous (Fig. 2B) and pyritic specimens (Fig. 4C). This likely is a combination of having a thicker wall when alive and more resistance to aerobic and anaerobic decay after death, the latter suggested by fewer attached saprophytic heterotrophs and EPSs. Taken together, these findings bear upon the extent to which the Schreiber biota was a primary, mat-building assemblage or a degradational assemblage, a question that long has been debated, with the paucity of aligned filaments and the presence of cysts suggesting the latter (18). Our data clearly point to a markedly degradational component.

Our combination of nanoscale isotopic and morphological analyses provides a powerful tool to assess the biogenicity of ancient pyritic objects and to distinguish biological from hydrothermal pyritization mechanisms. It also enhances the value of pyritic microfossils, demonstrating their importance as recorders of post-depositional biogeochemical processes throughout Earth's history.

Materials and Methods

Our samples come from a black stromatolitic chert from the lower 1.88-Ga Gunflint Formation that crops out along the northern shore of Lake Superior at the Schreiber Channel locality (14), close to Schreiber Beach. Samples for

transmission electron microscopy (TEM) were prepared using a dual-beam focused ion beam (FIB) system (FEI Helios NanoLab) at the Electron Microscopy Unit (EMU), University of New South Wales, and analyzed using a JEOL 2100 LaB6 TEM operating at 200 kV and a JEOL 3000F FEGTEM operating at 300 kV at the Centre for Microscopy, Characterization and Analysis (CMCA) at The University of Western Australia. Sequential FIB milling and scanning electron imaging were performed on a Zeiss Auriga CrossBeam dual-beam instrument at EMU, and 3D volume rendering was performed using the serial paleontological image editing and rendering system (SPIERS) software suite (33). Sulfur isotope ratios ($^{34}\text{S}/^{32}\text{S}$) from individual microfossils and groups of microfossils were determined at CMCA using the Cameca NanoSIMS 50 and Cameca IMS 1280 ion microprobes, respectively. The NanoSIMS 50 also was used for high-spatial resolution ion mapping of the microfossils. Laser Raman analyses were carried out at the University of Bergen using a Horiba LabRAM HR800 integrated confocal Raman system and LabSpec5 acquisition and analysis software. For detailed information on materials and methods see *SI Materials and Methods*.

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