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Peer Review File

A battle against arsenic toxicity by Earth's earliest complex life forms

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
This manuscript addresses a fundamental and intriguing question: the metabolism of the earliest eukaryotes and how it enabled them to persist beyond the GOE, an event linked to elevated arsenic levels in the marine environment. It seeks to compare arsenic levels and speciation inside and outside fossils dated to 2.1 Ga by conducting elemental analyses at both macroscopic and microscopic scales. Notably, the As-rich nuclei observed in biotic pyrite—absent in abiotic pyrites but also present in Phanerozoic eumetazoan pyrite—serve as compelling evidence for the biological origin of the Francevillian macrofossils.
The study places a strong emphasis on the environmental context, drawing upon elemental analyses to identify potential arsenic sources. It focuses on the elemental composition of geological units, particularly fossiliferous formations, as well as underlying strata.
The geochemical interpretation is tailored for a specialized audience and is supported by extensive references to relevant studies. However, the methodology underlying the sampling strategy for bulk analyses remains insufficiently detailed and would benefit from greater clarification.
The results suggest that arsenic found in the marine environment primarily originated from continental erosion. The hypothesis that As^{5+} was the dominant arsenic species in this setting is proposed, but documentation of the As^{5+}/As^{3+} ratio for these units should be provided to strengthen the argument.
Further investigations involve petrological analysis and trace element examination within the specimens. The interior of the fossils appears to be filled with pyrite, however establishing a clear spatial correlation between the regions where SEM images were collected and the corresponding specimen photographs remains challenging. Enhancing Figure 2 would aid in resolving this issue.
Elemental analyses were carried out directly on the pyrites. However, ambiguity persists regarding the exact number of specimens analyzed (the text implies that only two were examined), as well as the total number and type of samples, the number of measurements conducted, and their respective resolution.
Having established that the pyrites are enriched in arsenic, higher-resolution X-ray nanoimaging analyses were conducted to reveal zonations within macrofossil pyrites, particularly arsenic-enriched cores.
For nano-XRF measurements, details on beam operation mode, monochromator type, beam spot size, step increments, flux and on samples should be provided. Furthermore, supplementary material should include spectra from both the center and periphery of pyrite grains, along with their corresponding fits, to ensure that pile-up or other spectral interferences do not compromise the arsenic signal.
To thoroughly evaluate the XANES data, all aforementioned beam operation parameters should be explicitly stated, including the dwell time per point and the number of spectral repetitions. Were any assessments of radiation-induced alterations, such as photoreduction or oxidation, conducted during XANES measurements? Were any mitigation strategies implemented? By utilizing XANES reference spectra, it is possible to estimate the oxidation state of arsenic-bearing samples and compare findings between fossils and abiotic concretions, allowing for more definitive conclusions. The inclusion of such data would provide a more comprehensive framework for understanding arsenic speciation and its potential origins.
Finally, the discussion section appears disproportionately long compared to the remainder of the manuscript. A more concise and targeted approach, emphasizing the key findings, would significantly enhance the clarity and impact of the discussion.
The abstract should provide a clearer and more concise summary of the key findings.

Reviewer #3

(Remarks to the Author)
The manuscript focuses on the adaptation phenomena to the presence of toxic arsenic developed by the first multicellular life forms. It is an extremely interesting work that could appeal to a very wide audience, well beyond the scientific sphere. This work therefore seems to me to be appropriate in the chosen journal. The article is well written, easy to read, and the authors' reasoning is well explained. It is very educational and convincing. I have no complaints and I consider the article ready for acceptance, provided the other reviewers agree, of course.
Three easy remarks:
1. Two papers by Tribovillard (https://doi.org/10.1016/j.palaeo.2020.109745, and https://doi.org/10.1051/bsgf/2021034) on arsenic and antimony show that the enrichment factor calculations relative to the Earth's upper crust could have been slightly overestimated. In this case, this will not change the authors' interpretations, but it is worth clarifying to rule out any possible bias.
2. In Figure 1, the authors who originated the Al/(Al + Fe + Mn) vs. Fe/Ti diagram should be mentioned.
3. In Figure 2, it is a bit strange to speak of the Pyritized specimens of Gabon, as if everyone knew about the existence of these fossils, which are not (yet) as famous as the Ediacara faunas or the Burgess Shales!
NTribovillard

Reviewer 2 comments

This manuscript addresses a fundamental and intriguing question: the metabolism of the earliest eukaryotes and how it enabled them to persist beyond the GOE, an event linked to elevated arsenic levels in the marine environment. It seeks to compare arsenic levels and speciation inside and outside fossils dated to 2.1 Ga by conducting elemental analyses at both macroscopic and microscopic scales. Notably, the As-rich nuclei observed in biotic pyrite—absent in abiotic pyrites but also present in Phanerozoic eumetazoan pyrite—serve as compelling evidence for the biological origin of the Francevillian macrofossils. The study places a strong emphasis on the environmental context, drawing upon elemental analyses to identify potential arsenic sources. It focuses on the elemental composition of geological units, particularly fossiliferous formations, as well as underlying strata. The geochemical interpretation is tailored for a specialized audience and is supported by extensive references to relevant studies.

However, the methodology underlying the sampling strategy for bulk analyses remains insufficiently detailed and would benefit from greater clarification.

Response

We would like to take this opportunity to thank this reviewer for the supportive review.

We agree with this statement. Most bulk data were gathered from previously published work from Franceville (Aubineau et al., 2018, 2020; Bankole et al., 2020; Chi Fru et al., 2024). This information is included in the Materials and Methods: *"Some of these analyses in this study were previously treated and compiled*[33,35,37,92]*"*. The samples are representative of the different stratigraphic units described in the supplementary material (Section 1). Each sample was powdered using an agate mortar and approximately 1 g was fused with lithium metaborate ($LiBO_2$) and dissolved in dilute nitric acid (HNO_3). We have added these details in the Materials and Methods section.

The results suggest that arsenic found in the marine environment primarily originated from continental erosion. The hypothesis that As^{5+} was the dominant arsenic species in this setting is proposed, but documentation of the As^{5+}/As^{3+} ratio for these units should be provided to strengthen the argument.

Response

The reviewer raises an important point. As^{5+}/As^{3+} ratio in natural waters is primarily controlled by redox conditions, pH, and biological activity.

In well-oxygenated environments, arsenate (As^{5+}) is the dominant species, typically accounting for **90–100%** of total dissolved arsenic, due to the rapid oxidation and greater stability of As^{5+} under oxic conditions—particularly at circumneutral to alkaline pH (Cullen & Reimer, 1989; Smedley & Kinniburgh, 2002). Conversely, in anoxic, non-sulfidic settings, arsenite (As^{3+}) dominates, often comprising **80–100%** of total arsenic, with As^{5+} typically making up less than 1–20% (Cullen & Reimer, 1989; Smedley & Kinniburgh, 2002).

Our analysis establishes a well-defined redox profile across the studied succession. Based on this, we expect As^{5+} to prevail in oxic intervals, while As^{3+} dominates under anoxic, non-sulfidic conditions. In sulfidic settings, arsenic is expected to be sequestered predominantly as arsenic sulfide minerals.

This interpretation is supported by XANES analyses of Francevillian samples, which detected mainly As^{5+} and arsenic sulfides (Chi Fru et al., 2019). Similar findings from Chraiki et al. (2023) reinforce that As^{3+}-bearing minerals are rare in Precambrian marine sediments. This scarcity likely reflects the high mobility of As^{3+} in anoxic deep ocean waters, where it was subsequently mineralized into arsenic sulfides under sulfidic conditions—explaining their common occurrence in ancient anoxic sediments (Chi Fru et al., 2019).

In contrast, oxic settings—lacking sulfide mineral formation—favour the formation and preservation of As^{5+}. Therefore, we use the occurrence of As^{5+} and arsenic sulfides as reliable redox indicators to infer prevailing arsenic species in the depositional environment. This approach aligns with the modern consensus on redox-driven arsenic cycling (Cullen & Reimer, 1989; Smedley & Kinniburgh, 2002; Chi Fru et al., 2019; Chraiki et al., 2023).

We have clarified this rationale further in the revised text. We have added the following statement to the "Bulk rock As content and seawater trends*"* section in the discussion: *In well-oxygenated environments, As^{5+} is the dominant species, typically accounting for 90-100% of total dissolved As, due to rapid oxidation and higher stability than As^{3+}, particularly at circumneutral to alkaline pH*[45,46]*. Moreover, reconstruction of the oxidation state of As in marine shales showed a dominance of As-S and As^{5+} species, with As^{3+} being below the detection limit*[23]*.*

References

1. **Smedley & Kinniburgh. 2002.** A review of the source, behaviour and distribution of arsenic in natural waters. Applied Geochemistry 17, 517-568.
2. **Cullen W.R., Reimer K.J. 1989.** Arsenic speciation in the environment. Chemical Reviews 89, 13–764.
3. **Chraiki, I. *et al.* 2023.** Blooming of a microbial community in an Ediacaran extreme volcanic lake system. *Sci Rep* **13**, 9080.
4. **Chi Fru, E. C. *et al.* 2019**. The rise of oxygen-driven arsenic cycling at ca. 2.48 Ga. *Geology* **47**, 243–246.

Further investigations involve petrological analysis and trace element examination within the specimens. The interior of the fossils appears to be filled with pyrite, however establishing a clear spatial correlation between the regions where SEM images were collected and the corresponding specimen photographs remains challenging. Enhancing Figure 2 would aid in resolving this issue.

Response

We agree with this suggestion. A reannotation of images of Figure 2 has been done, pointing to the different parts of the fossils and their corresponding fillings by pyrite grains where necessary. The electron microscopy images show a vertical transect of the specimens. Figure 2 and its caption have been modified for more clarity.

Elemental analyses were carried out directly on the pyrites. However, ambiguity persists regarding the exact number of specimens analyzed (the text implies that only two were examined), as well as the total number and type of samples, the number of measurements conducted, and their respective resolution.

Response

We agree with this suggestion. The trace element concentrations were done on 10 samples (3 tubular fossils, 5 lobate fossils and 2 concretions). A total of 100 pyrite grains were measured (29 in tubulars, 51 in lobates and 20 in concretions). These details were added in the Materials and Methods section in the revised manuscript.

Having established that the pyrites are enriched in arsenic, higher-resolution X-ray nanoimaging analyses were conducted to reveal zonations within macrofossil pyrites, particularly arsenic-enriched cores. For nano-XRF measurements, details on beam operation mode, monochromator type, beam spot size, step increments, flux and on samples should be provided.

Response

We agree with the comment. Details of the X-ray nanoimaging and nano-XRF measurements have been provided in the Materials and Methods section as recommended. Below is the summary that has been inserted:

*"The SR nano-XRF and nano-XANES measurements were performed in multi-bunch beam operation mode on several areas within the studied samples at the NANOSCOPIUM hard X-ray nanoprobe beamline[97], Synchrotron SOLEIL, France. A Si(111) double-crystal fixed-exit monochromator was used for obtaining a monochromatic X-ray beam of 12 keV, just above the electron binding energy of the As K-edge. An achromatic nano-focusing Kirkpatrick-Baez (KB) mirror pair (JTEC) has been used for creating a 150 nm (FWHM) sized nano-beam at the sample position. Two silicon drift diodes detectors (SDD, VITUS H50, KETEK GmbH), situated symmetrically around the sample, were used for the measurements. The X-ray flux in the nano-beam has been adjusted in the [~5*108-5*109] photons/s range for each sample to keep the XRF detector's deadtime below 25% in order to optimize the quality of the XRF spectra. All scanning imaging were performed by using continuous sample scanning (FLYSCAN) developed at Synchrotron Soleil. The pixel size of the elemental distribution maps was in the 1 for coarse scans of mm-sized sample areas, except for the Phanerozoic fossils measured with (5 µm pixel size) shown in Figures S5 b-e, l-q, and in the 150 to 300 nm range for deca-µm2 scanned zones using 20-50 ms/pixel dwell times. An 8 µm thick silicon diode situated upstream of the KB-mirrors was used as intensity monitor to correct for the variation of the incident flux on the samples. In each measurement point (i.e. in each pixel for XRF imaging or in each energy step for XANES) we have*

collected full XRF spectra. The sum-spectra of the analysed sample areas have been fitted with the PyMCA software. The distribution maps of the elements identified from the sum-XRF-spectra were created either by PyMCA or by a homemade MATLAB code including corrections for deadtime, measurement time, beam intensity, and eventual spectral overlapping. Several areas have been studied for each sample. From the reconstructed elemental maps, the distributions of Fe, S, As, K, Ni, Cu and Ca were relevant for the recent study."

We have also included pixel size and dwel time in captions for Figures 4, 5 and 6.

Furthermore, supplementary material should include spectra from both the center and periphery of pyrite grains, along with their corresponding fits, to ensure that pile-up or other spectral interferences do not compromise the arsenic signal.

Response

We agree with this suggestion. An example of X-Ray Fluorescence spectra with corresponding elemental fits, one for the centre of a pyrite grain and another for the border, along with its corresponding pyrite grain and zone has been included in the supplementary material (Fig. S5). Moreover, Figs. S3 and S4 were added to show spectra for different zones chosen from two pyrite grains (one for a tubular specimen and another for a lobate). These data do not alter our interpretation.

To thoroughly evaluate the XANES data, all aforementioned beam operation parameters should be explicitly stated, including the dwell time per point and the number of spectral repetitions. Were any assessments of radiation-induced alterations, such as photoreduction or oxidation, conducted during XANES measurements? Were any mitigation strategies implemented? By utilizing XANES reference spectra, it is possible to estimate the oxidation state of arsenic-bearing samples and compare findings between fossils and abiotic concretions, allowing for more definitive conclusions. The inclusion of such data would provide a more comprehensive framework for understanding arsenic speciation and its potential origins.

Response

We agree with this suggestion. The omission of these details was an oversight from us. The complete XANES method was developed in the Materials and Methods section as follows: "*Nano X-ray absorption near edge structure spectroscopy (nano-XANES) has been performed in the very same measurement geometry as described above in nine As-rich pyrite nuclei in the macrofossils and three As hotspots in the pyritized concretions. The incident X-ray beam energy was scanned across the 11.825-11.910 keV energy rage in 0.5 eV increments and the data was collected in X-ray fluorescence (XRF) mode. The measurement time at each energy point was 1 s. Each XANES measurement in each measured sample point has been performed at least 3 times. These repetition scans showed no detectable photo-oxidation/photoreduction related alteration in the XANES spectra.*"

We have also changed figure 7 caption to enhance clarity.

Finally, the discussion section appears disproportionately long compared to the remainder of the manuscript. A more concise and targeted approach, emphasizing the key findings, would significantly enhance the clarity and impact of the discussion.

Response

We agree with the comment, but kindly request that the discussion remain largely as it is. This is for several reasons. The scientific community has consistently debated the validity of the Francevillian biota. To eliminate ambiguity, we provide sufficient context to substantiate our findings. This is essential, as the model we present is the first of its kind that will be published in the scientific literature, necessitating the integration of both modern literature and data to support our conclusions.

While the results are clear and focused, their broader implications require a more expansive discussion. For instance, we draw on existing literature to interpret bulk geochemical trends, explain arsenic-dependent pyrite nucleation, and justify why primitive macro-eukaryotes might sequester arsenic under the reconstructed environmental conditions by providing examples.

We believe the discussion, as written, offers the necessary depth, rationale, and references to communicate our findings effectively to a broad scientific audience—including geochemists, biologists, palaeontologists, and geobiologists. In conclusion, the discussion is structured to support the key points outlined in the abstract (see response to next comment) and conclusions.

The abstract should provide a clearer and more concise summary of the key findings.

Response

We agree with this comment. The abstract has been simplified further to emphasize our main findings and implications in the text, consisting of six clear points:

1. 2.1-billion-year-old Francevillian fossils are among the earliest complex life forms (statement to introduce the research)
2. These organisms lived in low-arsenic marine environments but show high arsenic levels in their bodies.
3. The arsenic was actively sequestered in specialized compartments, not due to post-mortem contamination.
4. Arsenic-rich pyrite nuclei indicate biological activity and support the fossils' biogenic origin.
5. Similar arsenic patterns are seen in later animals, but not in non-biological pyrite.
6. Early complex life developed strategies to cope with arsenic stress, even in low-arsenic settings (implications of our findings.

Reviewer 3 comments

The manuscript focuses on the adaptation phenomena to the presence of toxic arsenic developed by the first multicellular life forms. It is an extremely interesting work that could appeal to a very wide audience, well beyond the scientific sphere. This work therefore seems to me to be appropriate in the chosen journal. The article is well written, easy to read, and the authors' reasoning is well explained. It is very educational and convincing. I have no complaints and I consider the article ready for acceptance, provided the other reviewers agree, of course. Three easy remarks:

1. Two papers by Tribovillard (https://doi.org/10.1016/j.palaeo.2020.109745, and https://doi.org/10.1051/bsgf/2021034) on arsenic and antimony show that the enrichment factor calculations relative to the Earth's upper crust could have been slightly overestimated. In this case, this will not change the authors' interpretations, but it is worth clarifying to rule out any possible bias.

Response

We would like to thank this reviewer for the supportive review.

We agree with this comment. The statement: "*These enrichment calculations of As could slightly be overestimated due to a shuttle effect, but would affect all of the stratigraphic section*[40,41]" and the references have been included as recommended.

2. In Figure 1, the authors who originated the Al/(Al + Fe + Mn) vs. Fe/Ti diagram should be mentioned.

Response

We agree with this suggestion. The references have been added as suggested in Figure 1.

3. In Figure 2, it is a bit strange to speak of the Pyritized specimens of Gabon, as if everyone knew about the existence of these fossils, which are not (yet) as famous as the Ediacara faunas or the Burgess Shales! NTribovillard

Response

The reviewer raises an important point, however, pyritized specimens of Gabon are not related to fame but to the fact that the specimens are formed through the process of pyritization. This is similar to phosphatized and silicified fossils formed by phosphatization and silicification. In Gabon, there are pyritized and non-pyritized specimens that have been widely reported in the literature (e.g., El Albani et al., 2010, 2014).

References

1. **El Albani, A. *et al.*** Large colonial organisms with coordinated growth in oxygenated environments 2.1 Gyr ago. *Nature* 466, 100–104 (2010).

2. **El Albani, A. *et al*.** The 2.1 Ga Old Francevillian Biota: Biogenicity, Taphonomy and Biodiversity. *PLoS ONE* 9, e99438 (2014).