Response to Referee #1

General comment:

This manuscript entitled “Mineral formation induced by cable bacteria performing long-distance electron transport in marine sediments” investigates the nature of minerals associated with cable bacteria from different marine settings. Microscopy and spectroscopy analyses reveal (1) intracellular poly-phosphate accumulations in “naked” bacteria, (2) association of cells with extracellular clay minerals, most probably mediated by EPS and (3) different levels of encrustation by Fe-oxyhydroxides adsorbing phosphate.

The methodology is of quality, and the results are well discussed, without any excessive claim. These results are of high importance, as no previous study has been devoted to the analysis of minerals associated with these increasingly studied cable bacteria. They open wide and important issues to be explored. The manuscript is very well written, clearly organized and figures are of high quality. I recommend the publication of this manuscript in Biogeoscience, and have only minor comments.

Answer to general comment

We would like to thank this referee for the positive input and the very detailed comments which made it possible for us to dot the i’s and cross the t’s. We reply to each specific comment below.

Comment #1

In the introduction, you mention the fact that BIM leads to the formation of minerals that are indistinguishable of abiotic counterparts (l. 88-90). I would temper this claim, as actually, the simple fact that bacteria provide nucleation templates for mineral precipitation can lead to specific mineral textures (e.g. Mirvaux et al 2016) or even initiate mineral precipitation under conditions where this precipitation is kinetically hindered.

Answer to comment #1

We were not aware of this research, and we fully agree with the remark. We have changed the text as follows:

“Minerals that form by BIM generally nucleate and grow extracellularly as a result of the metabolic activity of the organism and subsequent chemical reactions involving metabolic by-products. BIM is an uncontrolled consequence of metabolic activity. The minerals formed are generally characterized by poor crystallinity, broad particle-size distributions, and lack of specific crystal morphology (Lowenstam and Weiner, 1989; Frankel and Bazylinsky, 2003). Both abiotic precipitation and BIM may result in minerals that are chemically and morphologically similar, though in other cases, there may be morphological differences. . This is because the bacterial surface provides nucleation templates for mineral precipitation, which act as a template for the growth and organization of the precipitated particles, thus leading to specific mineral textures (Mirvaux et al., 2016), or bacteria may initiate mineral precipitation under conditions where abiotic precipitation is kinetically hindered, which may also steer mineral morphology.”

Comment #2

I suggest to remove words like “unintended” (l. 86) or “inadvertent” (l. 538) that are not appropriate for the study of bacteria.

Answer to comment #2

In the revised text “unintended” is removed and “inadvertent” is changed to “uncontrolled”.

Comment #3

I suggest not to refer to poly-phosphate as a "mineral".

Answer to comment #3

We agree with this comment and will change the abstract to "…observed the formation of polyphosphate granules within the cells and two different types of mineral formation directly associated with multi-cellular filaments of these cable bacteria: the attachment of clay-particles into a coating surrounding the bacteria, and encrustation of the cell envelope by iron minerals."

Comment #4

I am not sure (unless for the study of iron encrustation) which conditions the samples were exposed to (oxic vs. anoxic). This deserves to be indicated as you prepared samples for microscopy through rinsing in non-degassed (i.e. oxic) water. In case some samples were initially under anoxic conditions, this method would likely induce the formation of secondary minerals (e.g. Fe oxyhydroxides). Please elucidate this point. In complement to this remark, I suggest the following modifications/discussions to be included:

- Add the site of collection and depth/condition (oxic vs. anoxic) in each figure legend.
- I wonder whether you observed different proportions of polyphosphates in samples collected in oxic vs. anoxic zones? This would be interesting to discuss if you have these informations, as part of a potential contribution of cable bacteria to the phosphorus cycle.

Answer to comment #4

This is a valuable remark, and we have will adapt the text to clarify differences (if any) between oxic vs. anoxic zones, and potential impacts of filament preparation.

In the many samples that we screened, there was no observation of Fe oxyhydroxides in samples from the anoxic zone, and so it is safe to assume that secondary Fe mineral formation did not occur despite the oxic conditions during sample preparation. Because individual cable bacteria or clumps of cable bacteria are washed through drops of de-ionized water there are no ions available for (secondary) mineral formation (e.g. there is ferrous iron to be oxidized). Only filaments extracted from the oxic zone showed Fe encrustation. This Fe encrustation is already observed under a light microscope right after the filaments are extracted from the sediment (before being rinsed with non-degassed water), and so the encrustation is formed *in situ*.

The sheaths with extracellular clay minerals observed around filaments were observed in both the oxic and suboxic zone and thus appears to form independent from the redox zonation. This sheath is already observed (albeit not as clearly as with electron microscopy) under a light microscope when the filaments are extracted from the sediment before being rinsed with non-degassed water.
We are aware that mineralogy might change during sample preparation. For now, we did not look at the mineral structure but elemental composition. When investigating the mineral structure (e.g. with STXM) this needs to be taken into account.

Concerning the polyphosphate granules we have only qualitative data, so for now we cannot elucidate if there are different proportions of polyphosphates in samples collected in the oxic vs. anoxic zones. To get more quantitative data nanoSIMS measurements or other small-scale techniques need to be performed that are not a part of this manuscript. The potential contribution of polyphosphates in cable bacteria to the phosphorus cycle has been investigated for Marine Lake Grevelingen and the

contribution was found to be negligible (Sulu-Gambari et al., 2016). Research on a more spatial and temporal scale would be useful but is not a part of this manuscript.

For clarity, we added the site of collection and the condition from where the sample was taken to the legends.

Comment #5

Discussion:

- Around l. 420: regarding the possibility to evaluate the similarity between poly-P granules and acidocalcisomes, you could also mention the use of fluorescent pH-probes as a potential method.
- L. 467: regarding acidic micro-environments, you could instead or in addition cite: (Hegler *et al*., 2010).
- L. 474 and l. 543: For the discussion about the impact of encrustation on metabolic activity, (Miot *et al.*, 2015) have quantified the impact of the level of Fe(III)-mineral encrustation on the uptake of organic molecules (acetate) in Fe(II)-oxidizing bacteria (BoFeN1). This should be discussed here. As an additional strategy to avoid cell encrustation (at the population level), you can thus mention the co-existence of bacteria at different levels of encrustation with the naked cable bacteria. Indeed, you mention that you systematically observe non-encrusted cells, which is consistent with observations by Miot et al (2015) with Fe(II)-oxidizing bacteria.
- L. 502-503: the nature of the minerals could be indeed evaluated by STXM, but also by TEM.
- The FIB-SEM images/videos are very impressive! However, you should mention somewhere in the discussion the potential artifacts that may be induced by the preparation (resin embedding) and analysis of the sample (e.g. (Dohnalkova *et al.*, 2011; Miot *et al.,* 2011; Bassim *et al*., 2012). Cryo-methods could very interestingly complement your observations.
- You could summarize more clearly the potential role of cable bacteria in Fe-mineral formation: (1) their surface can provide a nucleation site for mineral precipitation, (2) their metabolism (in particular O_2 respiration in the oxic zone) may locally increase the pH (local pH gradient around the cells), which would be favorable to Fe-mineral precipitation and growth. The nano-sized globules observed on some cells could correspond to early stages of cell encrustation.

Answer to comment #5

- We will add the use of fluorescent pH-probe as a potential method and use Brock et al., 2012 and Hegler et al, 2010 as a reference.
- Hegler et al, 2010 has now been cited in addition to Schadler et al, 2009. Thank you. We were not aware of this publication.
- We will add a sentence after l. 474:
- “Cell encrustation could potentially limit the diffusion of substrates and nutrients to the cell, impair uptake of these compounds across the membrane, and as a consequence lead to the stagnation of cell metabolism and eventually even to cell death (Konhauser, 1998b; Schädler et al., 2009). When a culture of the Fe(II) oxidizing bacteria *Acidovorax* sp. strain Bo1FeN1 was exposed to high concentrations of Fe^{2+}, most cells became encrusted with iron minerals. Cells that were moderately encrusted still had the capacity to assimilate acetate but with increasing levels of iron encrustation the capacity to assimilate carbon decreased exponentially. Remarkably, a small proportion of cells remained free of encrustation and metabolically active implying that phenotypic heterogeneity might be a viable strategy to cope with biomineralization (Miot et al., 2015). Since both encrusted and non-encrusted filaments co-exist (Fig. 11a) this strategy might also be employed by cable bacteria.”

- TEM was added as a method.
- We are aware that the sample preparation method may have resulted in the loss of microstructures and a change in cell morphology. However, the FIB-SEM videos were used to analyze the thickness and structure of the encrustation and to identify the location of the biomineral layer, which could be on the outside of the filament , or alternatively, within the periplasmic space underneath the ridge structure. In the FIB-SEM images/videos periplasmic space is visible and therefore the possible loss of microstructures or changes in the chemical composition would not change the conclusion that the biomineral formation took place at the outside of the mineral structure. Potential artifacts would be a problem when looking at the chemical interaction between the outer cell surface, EPS and the mineral layer. For this, cryogenic methods appear to be the most promising since they would preserve the native structure. As a suggestion for further research we have added a sentence after l. 506 where cryogenic methods are mentioned and the suggested publications were cited:
  "To further investigate the interaction between the outer cell surface, EPS and the mineral layer, cryogenic methods appear to be promising since they would preserve the native structure (Bassim et al., 2012; Dohnalkove et al., 2011; Miot et al., 2011b)"
- To articulate the potential role of cable bacteria in Fe biomineral formation we will rewrite the last paragraph of the discussion as follows:
  "It appears that the metabolism of cable bacteria results in a cascade of reactions that eventually results in the uncontrolled mineralization of filaments that are present in the oxic zone. The cell surface provides a nucleation site and template for mineral formation, and the increase of the pH in the oxic zone as a result of the electrogenic metabolism of cable bacteria, favors Fe-mineral precipitation and growth. Since the mineral precipation does not appear to be controlled by the cable bacteria, it forms an example of biologically induced mineralization. The formation of a mineral crust on a cell surface could potentially limit cell metabolism and may eventually lead to cell death (Konhauser, 1998a; Schädler et al., 2009). However, the extent to which this affects cable bacteria is currently unknown and so the impact of encrustation on cable bacteria metabolism needs to be resolved."

Minor corrections:

- Replace the wording "microscopic techniques" by "microscopy techniques" (e.g. l. 21, 103)
- Materials and methods (l. 215 and next): indicate the modes of SEM imaging (backscattered vs secondary electron mode). This should be mentioned in figure legends as well if different modes have been applied.
- Fig. 4: mistake in the legend. Add (i): P and S…..
- L. 400-401: "The storage of P […] the compounds". I do not understand this sentence.

Answer to minor corrections:

All minor corrections will be changed in the final manuscript. We would like to thank the reviewer for the attention to detail.

L. 400-401 will be rewritten and incorporated with l. 393-395: "Acidocalcisomes are an electron-dense, acidic compartment containing a matrix of pyrophosphate and polyphosphates with bound calcium and other cations, mainly magnesium and potassium. The formation of acidocalcisomes hence allows increased uptake of both phosphorus compounds and cations (Docampo and Moreno, 2012)."