Necessity of electrically conductive pili for methanogenesis with magnetite stimulation

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Background: Magnetite-mediated direct interspecies electron transfer (DIET) between *Geobacter* and *Methanosarcina* species is increasingly being invoked to explain magnetite stimulation of methane production in anaerobic soils and sediments. Although magnetite-mediated DIET has been documented in defined co-cultures reducing fumarate or nitrate as the electron acceptor, the effects of magnetite have only been inferred in methanogenic systems. **Methods:** Concentrations of methane and organic acid were analysed with gas chromatograph and high-performance liquid chromatography, respectively. The concentration of HCl-extractable Fe(II) was determined by the ferrozine method. The association of the defined co-cultures of *G. metallireducens* and *M. barkeri* with magnetite was observed with transmission electron micrographs. **Results:** Magnetite stimulated ethanol metabolism and methane production in defined co-cultures of *G. metallireducens* and *M. barkeri*; however, magnetite did not promote methane production in co-cultures initiated with a culture of *G. metallireducens* that could not produce electrically conductive pili (e-pili), unlike the conductive carbon materials that facilitate DIET in the absence of e-pili. Transmission electron microscopy revealed that *G. metallireducens* and *M. barkeri* were closely associated when magnetite was present, as previously observed in *G. metallireducens/G. sulfurreducens* co-cultures. These results show that magnetite can promote DIET between *Geobacter* and *Methanosarcina* species, but not as a substitute for e-pili, and probably functions to facilitate electron transfer from the e-pili to *Methanosarcina*. **Conclusion:** In summary, the e-pili are necessary for the stimulation of not only *G. metallireducens/G. sulfurreducens*, but also methanogenic *G. metallireducens/M. barkeri co-cultures* with magnetite.

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Running title: E-pili for magnetite stimulation

ABSTRACT

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Methods: Concentrations of methane and organic acid were analysed with gas chromatograph and high-performance liquid chromatography, respectively. The concentration of HCl-extractable Fe(II) was determined by the ferrozine method.

The association of the defined co-cultures of *G. metallireducens* and *M. barkeri* with magnetite was observed with transmission electron micrographs.

Results: Magnetite stimulated ethanol metabolism and methane production in defined co-cultures of *G. metallireducens* and *M. barkeri*; however, magnetite did not promote methane production in co-cultures initiated with a culture of *G. metallireducens* that could not produce electrically conductive pili (e-pili), unlike the conductive carbon materials that facilitate DIET in the absence of e-pili. Transmission electron microscopy revealed that *G. metallireducens* and *M. barkeri* were closely associated when magnetite was present, as previously observed in *G. metallireducens/G. sulfurreducens* co-cultures. These results show that magnetite can promote DIET between *Geobacter* and *Methanosarcina* species, but not as a substitute for e-pili, and probably functions to facilitate electron transfer from the e-pili to *Methanosarcina*.

Conclusion: In summary, the e-pili are necessary for the stimulation of not only *G. metallireducens/G. sulfurreducens*, but also methanogenic *G. metallireducens/M. barkeri co-cultures* with magnetite.

INTRODUCTION

Microbial methane production is one of the most successful, large-scale bioenergy strategies (*Liu et al*., *2009*; *Shen et al*., *2016*) and methane production in terrestrial environments is a major source of atmospheric methane, an important greenhouse gas (*Bridgham et al*., *2013*; *Conrad 2007*). In freshwater methanogenic environments, and anaerobic digesters, methanogens primarily produce methane from the metabolism of acetate and the reduction of carbon dioxide with H_2 to methane. The well-known source of electrons for carbon dioxide reduction to methane is H_2 (*Sieber et al*., *2012*); however, it has recently been demonstrated that *Methanosaeta* and *Methanosarcina* species can accept electrons from the donor strain *G. metallireducens* for carbon dioxide reduction via direct interspecies electron transfer (DIET) (*Chen et al*., *2014a*; *Chen et al*., *2014b*; *Rotaru et al*., *2014a*; *Rotaru et al*., *2014b*; *Wang et al*., *2016*).

In the absence of added conductive materials, DIET between *Geobacter metallireducens* and *Methanosaeta* and *Methanosarcina* species requires the electrically conductive pili (e-pili) of *G. metallireducens* (*Chen et al*., *2014a*; *Rotaru et al*., *2014a*; *Rotaru et al*., *2014b*). The e-pili of both *Geobacter* species are also required for DIET in co-cultures of *G. metallireducens* and *G. sulfurreducens* (*Shrestha et al*., *2009*; *Summers et al*., *2010*). Existing studies on the e-pili of *G. sulfurreducens* have suggested that the conductivity along the length of *Geobacter* e-pili (*Adhikari et al*., *2016*; *Malvankar & Lovley 2014*) can be attributed to the tight packing of aromatic amino acids within the pilus structure, which confer a metallic-like conductivity similar to that observed in carbon nanotubes (*Malvankar et al*., *2015*; *Malvankar et al*., *2011*; *Vargas et al*., *2013*). The e-pili are decorated with the *c*-type cytochrome OmcS, which does not contribute to conductivity along the length of the e-pili, but is important for electron transfer from the e-pili to extracellular electron acceptors/donors (*Leang et al*., *2010*; *Liu et al*., *2015*; *Malvankar & Lovley 2014*; *Malvankar et a*l., *2012*; *Mehta et al*., *2005*; *Summers et al*., *2010*). It is expected that the e-pili of *G. metallireducens* function in a similar manner(*Smith et al*., *2013*; *Tremblay et al*., *2012*; *Zheng et al*., *2017*), but the cytochrome(s) that are attached to the e-pili of *G. metallireducens* have not yet been identified.

Conductive carbon materials, such as: granular activated carbon, carbon cloth, and biochar, stimulate DIET *(Chen et al*., *2014a*; *Chen et al*., *2014b*; *Liu et al*., *2012*; *Rotaru et al*., *2014a*). The electron-donating and electron-accepting partners both attach to the conductive carbon materials, which serve as an electrical conduit between the two species. Mutant *Geobacter* strains that lack e-pili can participate in DIET under these conditions, presumably because biological cell-to-cell electrical conduits are no longer required (*Chen et al*., *2014a*; *Chen et al*., *2014b*; *Liu et al*., *2012*; *Rotaru et al*., *2014a*).

An important insight into carbon and electron flow in methanogenic environments lies in the finding that magnetite stimulated methane production in enrichment cultures inoculated from paddy soil with either ethanol or acetate as the electron donor (*Kato et al*., *2012a*). The enhanced methane production was accompanied by an enrichment of *Geobacter* and *Methanosarcina* species (*Kato et al*., *2012a*). It was hypothesised that the magnetite provided electrical contact between the *Geobacter* and *Methanosarcina* species and that the *Geobacter* species oxidized the ethanol or acetate to carbon dioxide with electron transfer to the *Methanosarcina*, which then used the electrons to reduce carbon dioxide to methane (*Kato et al*., *2012a*). Many subsequent studies have documented the fact that magnetite accelerates methane production in samples from sediments or anaerobic digesters or defined co-cultures and have also inferred that this can be attributed to enhanced electron transfer through magnetite to methanogens (*Li et al*., *2015*; *Tang et al*., *2016*; *Yang et al*., *2015*; *Zhuang et al*., *2015*). Magnetite does promote interspecies electron transfer between *Geobacter sulfurreducens* and *Thiobacillus denitrificans* growing with acetate as the electron donor and nitrate as the electron acceptor (*Kato et al*., *2012b*), as well as between *G. metallirducens* and *G. sulfurreducens* growing with ethanol as the electron donor and fumarate as the electron acceptor (*Liu et al*., *2015*)., however, it has never been directly demonstrated that magnetite promotes DIET to methanogens. Analysis of the mechanisms by which magnetite enhanced DIET in *G. metallireducens/G. sulfurreducens* co-cultures indicated that, unlike conductive carbon materials, magnetite does not act as a substitute for e-pili, but

rather can take the place of OmcS by attaching to e-pili to facilitate DIET, thus alleviating the need for OmcS production (*Liu et al*., *2015*). Therefore, it should not be assumed that magnetite promotes DIET to methanogens as has been demonstrated for conductive carbon materials. The purpose of this study was to evaluate further the possibility that magnetite promotes DIET to methanogens.

MATERIALS AND METHODS

Microorganisms, media, and growth conditions

Wild-type *Geobacter metallireducens* strain GS-15 (ATCC 53774) (*Aklujkar et al*., *2009*; *Lovley et al*., *1993*) and a strain of *G. metallireducens* in which the gene for PilA, the pilus monomer, was deleted (*Tremblay et al*., *2012*) were obtained from our laboratory collection. *Methanosarcina barkeri* strain DSM 800 (ATCC 43569) was obtained from DSMZ (Braunschweig, Germany).

All culturing was performed under strict anaerobic conditions under a gas phase of N_2/CO_2 (80/20). Inocula for co-cultures were developed by growing *G. metallireducens* strains in Fe(III)-citrate (FC) medium (*Bagnara et al*., *1985*), with 20 mmol L^{-1} ethanol as the sole electron donor and 55 mmol L^{-1} Fe(III) citrate as the electron acceptor. For co-cultures of *G. metallireducens* and *M. barkeri*, *G. metallireducens* was grown in DSMZ methanogenic medium 120 with 20 mmol L^{-1} ethanol as the electron donor and nitrate (10 mmol L^{-1}) as the electron acceptor. *M. barkeri* was grown in the same medium with 30 mmol L^{-1} acetate as the substrate. Co-cultures were grown in 40 mL medium 120 with a 10% inoculum and with ethanol (20 mmol L^{-1}) as the electron donor as described previously (*Rotaru et al*., *2014a*). The incubation temperature for all methanogenic studies was 37 °C. When noted, magnetite was prepared as previously described (*Kang et al*., *1996*) and added from stock solutions to give a final concentration of 5 mmol L^{-1} before autoclaving.

Chemical analysis

The gaseous samples were regularly collected from enrichment cultures with pressure-lock analytical syringes. The concentrations of CH_4 were analysed with a gas chromatograph (GC-7890A; Agilent Technologies, USA) equipped with a flame ionisation detector.

Concentrations of ethanol and acetate were analysed with high-performance liquid chromatography (1260 Infinity; Agilent Technologies, USA) with a Hi-plex H column equipped with a refractive index detector.

The concentration of HCl-extractable Fe(II) was extracted from cultures and each replicate of the assays in triplicate as described previously (*Zheng et al*., *2015*). Moreover, the concentration of dissolved Fe(II) in samples was also quantified by filtering through 0.45 μm sterile syringe filters and using the ferrozine method as described above.

Microscopy

Samples of cells and magnetite were negatively stained with 2 % phosphotungstic acid and examined by a JEM-1400 (JEOL, Japan) transmission electron microscope (TEM).

RESULTS AND DISCUSSION

Magnetite stimulation of DIET between *G. metallireducens* and *M. barkeri*

To evaluate whether, or not, magnetite was capable of stimulating DIET between *G. metallireducens* and *M. barkeri*, co-cultures were initiated with ethanol as the sole electron donor in the presence, and absence, of magnetite. Although *M. bakeri* is capable of using H_2 as an electron donor, *G. metallireducens* cannot metabolise ethanol with the production of H_2 (*Rotaru et al*., *2014b*; *Shrestha et al*., *2013a*; *Summers et al*., *2010*) and thus syntrophic growth in *G. metallireducens/M. barkeri* co-cultures can be attributed to DIET.

The initial establishment of *G. metallireducens* and *M. barkeri* co-cultures requires a long adaption period in the absence of added conductive materials (*Rotaru et al*., *2014a*). As expected, ethanol was only slowly metabolised over 50 days without magnetite (Fig. 1C), however, in the

145 presence of magnetite, ethanol was metabolised with the production of methane beginning within
146 10 days (Fig. 1C). Non-inoculated controls with magnetite showed no ethanol metabolism or
147 methane production.

148 Limited acetate accumulated in the *G. metallireducens* with, or without, *M. barkeri* co-
149 cultures in the presence of magnetite ($C_2H_6O + H_2O \rightarrow C_2H_4O_2 + 4H^+ + 4e^-$, Oxidation of one
150 ethanol will produce one acetate plus four electrons released (*Rotaru et al., 2014a*)), but was
151 later consumed (Fig. 1D), which differed from co-cultures of *G. metallireducens* and *M. barkeri*
152 in the absence of magnetite, suggesting that *G. metallireducens* metabolised the acetate that *G.*
153 *metallireducens* produced from ethanol compared with the result of *G. metallireducens* acting
154 alone with magnetite. The high amount of methane in the *G. metallireducens* and *M. barkeri* co-
155 cultures suggested that *M. barkeri* only used the electrons released from ethanol oxidation for
156 reducing carbon dioxide to produce methane in the magnetite-amended co-cultures ($8H^+ + 8e^- +$
157 $CO_2 \rightarrow CH_4 + 2H_2O$). The total amount of ethanol from the magnetite-amended co-cultures
158 metabolised (1.15 ± 0.12 mmol) resulted in 1.60 ± 0.0032 mmol methane (Figs 1A, C), which
159 showed that the mmol ratio of CH_4/C_2H_6O (1.60/1.15) was 1.39 (＞1), thus about 92.2 % of the
160 electrons from ethanol oxidation were recovered in methane according to the equation: $2C_2H_5OH$
161 $\rightarrow 3CH_4 + CO_2$. Furthermore, no H_2 was detected in any of the experiment groups. This result
162 was consistent with the fact that *G. metallireducens* is unable to produce H_2 during ethanol
163 metabolism (*Shrestha et al*., *2013b*). Therefore, the high electron recovery that was available
164 from ethanol to methane suggested that magnetite can stimulate DIET between *G.*
165 *metallireducens* and *M. barkeri* and suggested that the simplest explanation for the enrichment of
166 *Geobacter* and *Methanosarcina* observed in the presence of magnetite in previous studies (*Kato*
167 *et al*., *2012a*) is that magnetite was facilitating DIET.

168 HCl-extractable ferrous iron was also produced from reduction ferric iron of magnetite
169 within five days and increased to 0.1768 ± 0.0219 mmol at 50 days, which was equal to that
170 when *G. metallireducens* was tested with magnetite alone (0.1761 ± 0.0549 mmol) (Fig. 1B);

however, the concentration of dissolved ferrous iron was under detect limitation during the incubation of the co-cultures amended with magnetite, suggested that only a part of the ferric iron in the added magnetite was reduced to ferrous iron. The results indicated that only a small portion of electrons were to ferric iron reduction and the majority of electrons were to methane production. This result differs from that reporting that magnetite acts as the electrical conduit between electron-donating *Geobacter* and electron-accepting methanogens (*Kato et al*., *2012a*; *Li et al*., *2015*; *Viggi et al*., *2014*). One factor controlling ferrous iron production in co-cultures amended with magnetite is the range of substrates that can be metabolised by *Geobacter* species. *G*. *metallireducens* can utilise ethanol and acetate, ferrous iron production from acetate was slower than that from ethanol within 10 days in the presence of magnetite, while ferrous iron production from *G. sulfurreducens* amended with magnetite was much lower than that from *G*. *metallireducens* when utilising acetate (Fig. 2). This suggested that *G*. *metallireducens*, like some microorganisms (*e.g*., *Shewanella*, *Dechloromonas*, *Desulfovibrio*, and *Clostridium*) was able to use magnetite as the electron acceptor from ethanol or acetate metabolism (*Kostka & Nealson 1995*; *Yang et al*., *2015*). However, it is not possible for magnetite to act as the electron shuttle for production of methane from carbon dioxide because of the relatively high mid-point potential of the Fe(III)/Fe(II) redox couple (E_0' = +0.20 V, pH 7.0) which is too high for the reduction of carbon dioxide to methane (E_0' of CO_2/methane couple = -240 mV).

To determine the actual role of magnetite in stimulation of ethanol metabolism and methane production in co-cultures of wild-type *G*. *metallireducens* and *M. barkeri*, co-cultures were initiated with 2.5 mmol L^{-1} magnetite, after a 10-day incubation period, an additional 2.5 mmol L^{-1} magnetite was subsequently added. Methane production presented the same tendency with 5 mmol L^{-1} magnetite added to the co-cultures (Fig. 1A): this meant that the manner and amount of addition of magnetite could not affect methane production, however, the amount of HCl-extractable ferrous iron changed: the reduced ferrous iron concentration was about 0.0193-0.0239 mmol (~9.6-12% of added Fe^{3+}) when 2.5 mmol L^{-1} magnetite (Fe^{3+}: 0.2 mmol) was added

during the first 10 days, and subsequently reduced when more magnetite was added, the amount of ferrous iron used in each step (total: 0.1635 ± 0.0313 mmol) was similar to the addition of 5 mmol L^{-1} magnetite (Fig. 1B). This result was consistent with the observation that *G. metallireducens* alone reduced magnetite to produce ferrous iron (Fig. 1B). Thus, the initial concentration of magnetite determined how much Fe(III) inside was reduced. When high concentration of magnetite (5 mmol L^{-1}) was available, Fe(III) reduction was detected; however, no significant Fe(III) reduction was found when lower concentration of magnetite (2.5 mmol L^{-1}) was present. Fe(III) in the magnetite was reduced only when additional magnetite (2.5 mmol L^{-1}) was added. This demonstrated that lower concentration of magnetite could not be preferentially used as the electron acceptor in the co-culture of *G. metallireducens* and *M. barkeri*. Similarly, ethanol was stimulated to oxidise and little acetate was transiently accumulated in magnetite upon its step-by-step addition to co-cultures of wild-type *G. metallireducens* and *M. barkeri* (Figs 1C, D). The calculation of electron recovery (93.81%) of electrons available from ethanol in methane in these samples further suggested that *M. barkeri* was accepting electrons from carbon dioxide reduction via DIET.

Transmission electron microscopy (TEM) revealed that *G. metallireducens* (rod-shaped cells) and *M. barkeri* (larger size cocci) were associated with each other (Fig. 3A). With higher magnification it was apparent that magnetite was associated with the *G. metallireducens* pili (Fig. 3B), as was previously observed that some of the magnetite was localised along pili and compensated for the lack of OmcS of *G. sulfurreducens* in promoting electrical contacts with pili in *G. metallireducens/G. sulfurreducens* co-cultures (Liu et al. 2015).

Failure of magnetite to compensate for loss of e-pili in *G. metallireducens*

To investigate further the mechanisms for magnetite stimulation of DIET between *G. metallireducens* and *M. barkeri*, co-cultures were initiated with the previously described strain of *G. metallireducens* (*Tremblay et al*., *2012*) that is incapable of producing pili because the gene for PilA, the pilus monomer, has been deleted. As expected from previous studies (*Rotaru et al*.,

2014a), methane was not produced in co-cultures with the pili-deficient strain of *G. metallireducens* (Fig. 4A), however, co-cultures amended with magnetite produced less methane (about 0.38 ± 0.025 mmol, Fig. 4A). During co-culture testing, ferrous iron concentrations were below 0.1 mmol in magnetite amended cultures (Fig. 4B). Furthermore, co-cultures with the *pil*A-deficient *G. metallireducens* failed to metabolise ethanol or produce acetate with, or without, magnetite amendment (Figs 4C, D). These results suggested that magnetite perhaps can partly substitute for pili to participate in DIET of co-cultures resulting from its electrical conductivity; however, magnetite appears to promote DIET by a mechanism that is different than that in conductive carbon materials such as GAC and carbon cloth (*Chen et al*., *2014a*; *Liu et al*., *2012*). In the presence of GAC or carbon cloth the pili-deficient strain of *G. metallireducens* can transfer electrons to *M. barkeri* because both species attach to the conductive materials, which are much bigger than individual cells. Magnetite particles are typically smaller (at 20-50 nm) than cells and thus are unlikely to provide effective cell-to-cell contacts (*Liu et al*., *2015*). This was evident in previous studies with *G. metallireducens/G. sulfurreducens* co-cultures in which magnetite was not able to compensate for the lack of e-pili in *G. metallireducens* (*Liu et al*., *2015)*. Multiple lines of evidence, including studies with an OmcS-deficient mutant, suggested that magnetite could serve as the functional equivalent of OmcS, and the *c*-type cytochrome associated with the e-pili of *G. sulfurreducens* (*Liu et al*., *2015*). Similar genetic experiments are not yet possible with *G. metallireducens* because the cytochrome(s) associated with the *G. meatllireducens* e-pili have not been identified. However, the finding that magnetite amendments did not permit the growth of the pili-deficient strain of *G. metallireducens* in co-culture with *M. barkeri*, suggested the magnetite cannot function as an e-pili substitute in all regards. Magnetite was associated with the e-pili in the *G. metallireducens/M. barkeri* co-cultures. Therefore, it is likely that magnetite also facilitated electron transfer from the *G. metallireducens* e-pili to *M. barkeri* in the co-cultures.

CONCLUSIONS

In sum, we have established co-cultures of *M. barkeri* and wild-type *G. metallireducens* or a strain deficient in the PilA gene with or without magnetite. The results revealed magnetite stimulated ethanol metabolism and methane production in defined co-cultures of *G. metallireducens* and *M. barkeri*. However, magnetite did not promote methane production in co-cultures of the *pilA*-deficient *G. metallireducens.* These results showed that magnetite could not substitute for e-pili to promote DIET between *Geobacter* and *Methanosarcina* species, in which the e-pili are necessary for the stimulation.

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380

Figure 1(on next page)

Co-cultures of *G. metallireducens* (*G. m*) and *M. barkeri* (*M. b*) with ethanol as the substrate in the presence, or absence, of magnetite (Fe_3O_4).

Quantities of methane (A), ferrous iron (B), ethanol(C), and acetate (D) in cultures. Data are the means and standard deviation for triplicate cultures. In some instances the standard deviation was less than the size of the symbol.

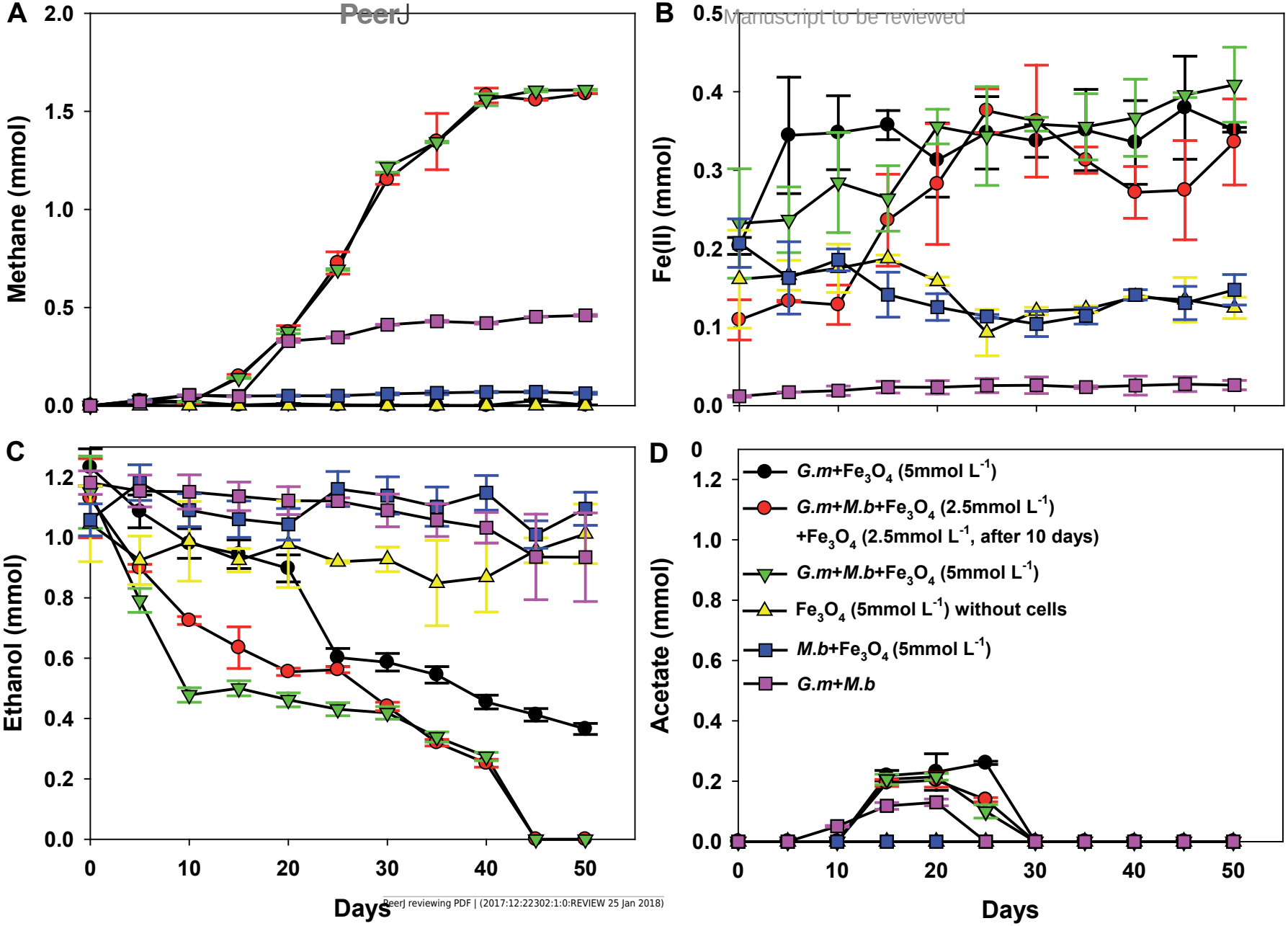


Figure 2(on next page)

Quantities of ferrous iron in cultures of *Geobacter metallireducens* (*G.m*) and *Geobacter sulfurreducens* (*G.s*) in the presence of magnetite with ethanol and acetate as the substrates.

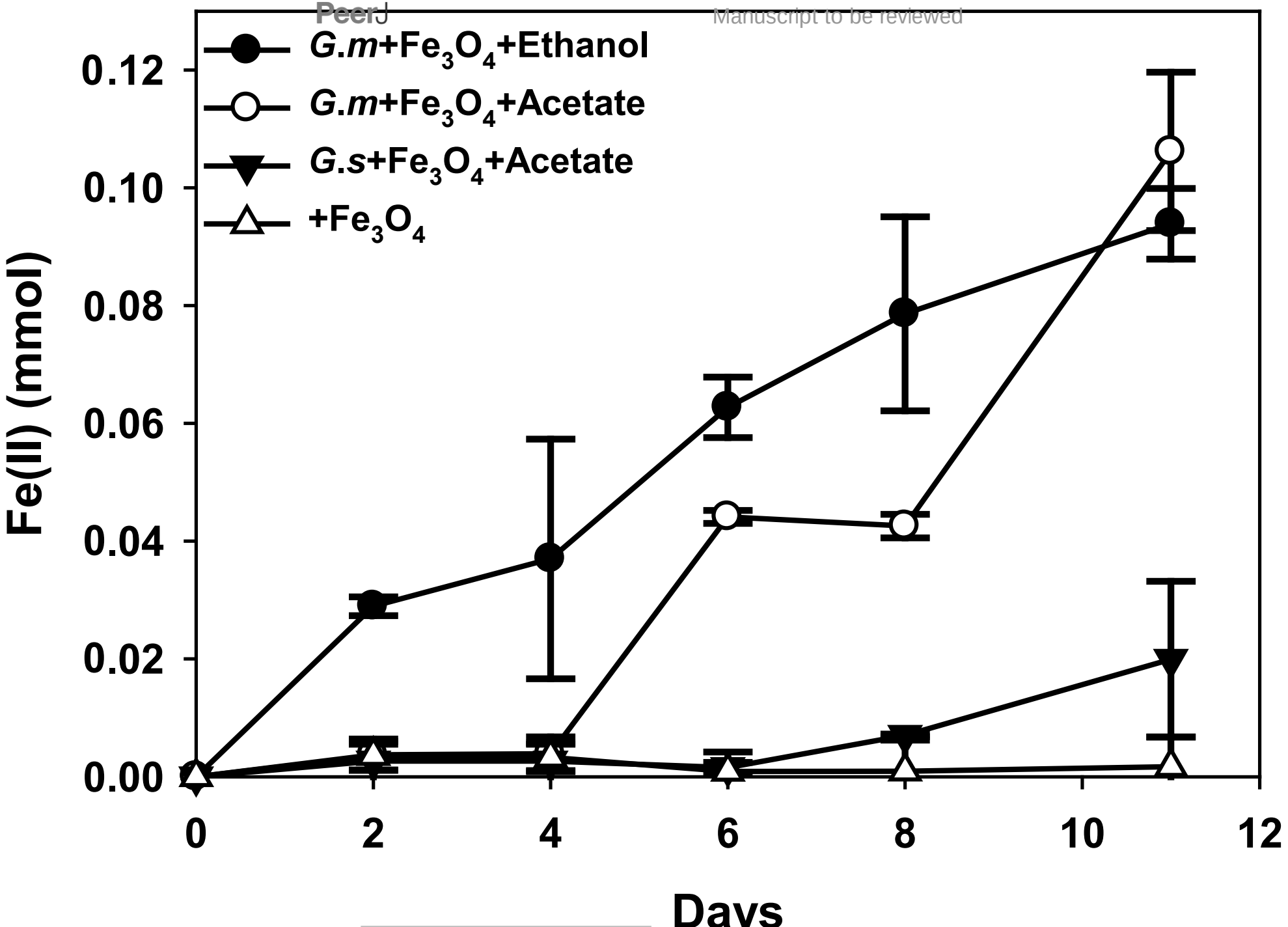
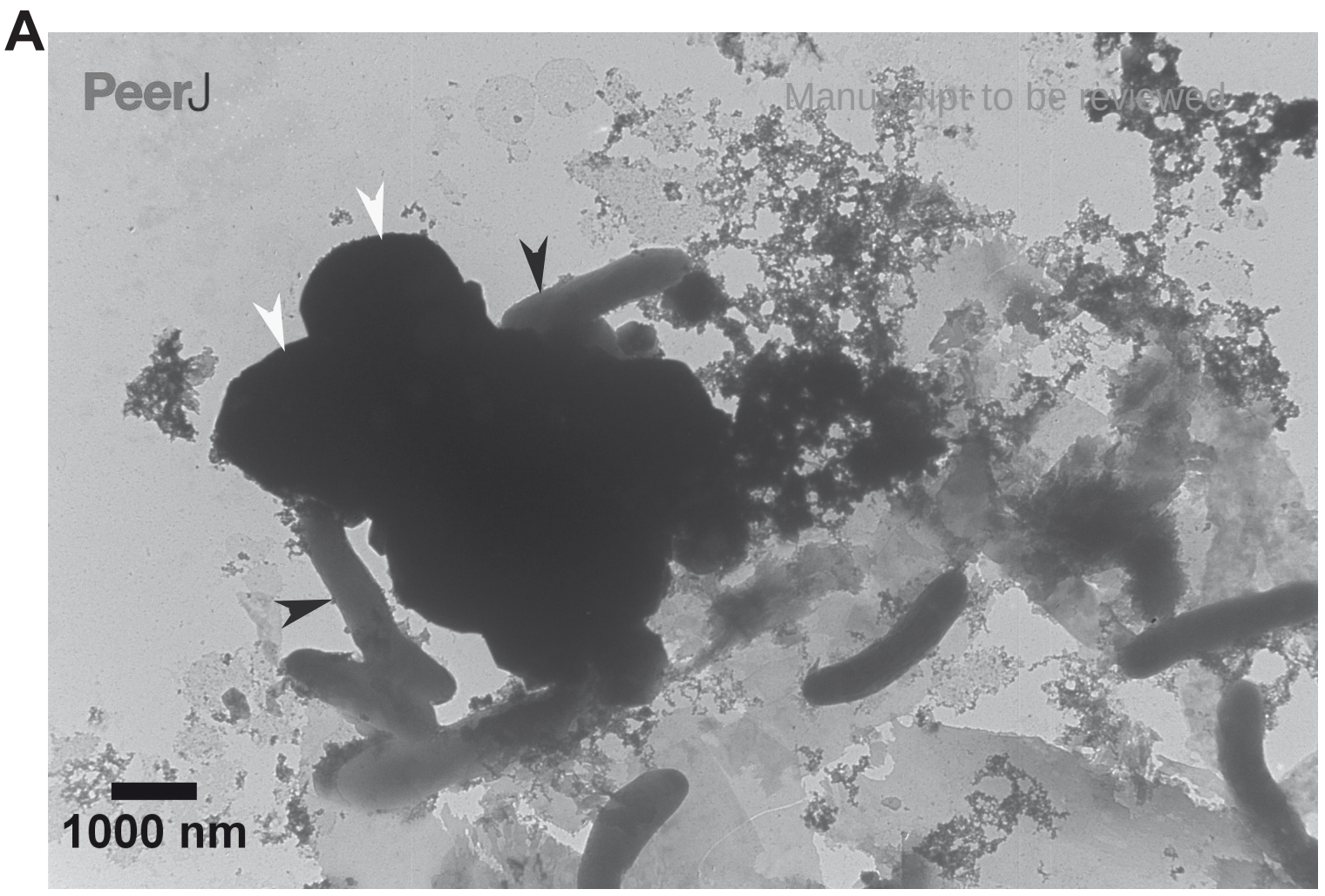

G.m+Fe3O4+Ethanol
G.m+Fe3O4+Acetate
G.s+Fe3O4+Acetate
+Fe3O4
Fe(II) (mmol)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0
2
4
6
8
10
12
Days

Figure 3(on next page)

Transmission electron micrographs.

Association of the defined co-cultures of G. metallireducens and *M. barkeri* with magnetite. (A) Association of the two cell types. Black and white arrows indicate *G. metallireducens* cells and *M. barkeri* cells, respectively. (B) Association of magnetite with pili. White arrows indicate magnetite and pili.



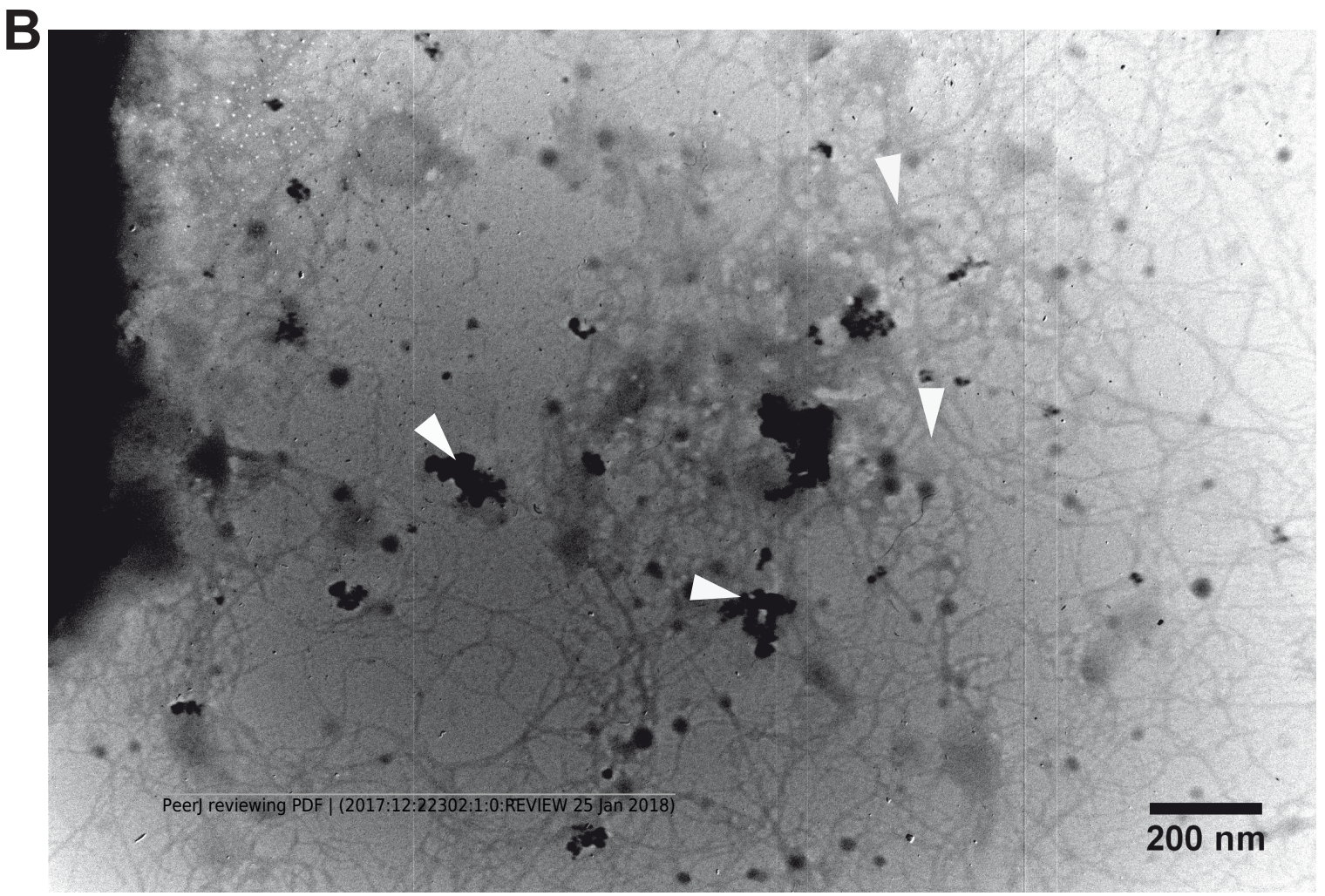


Figure 4(on next page)

Co-cultures of *M. barkeri* (*M. b*) and a *PilA-deficient G.metallireducens* (*G. m-deltapilA*) strain in the presence, or absence, of magnetite (Fe_3O_4).

Quantities of methane (A), ferrous iron (B), ethanol(C), and acetate (D) in cultures. Data are the means and standard deviation for triplicate cultures. In some instances the standard deviation was less than the size of the symbol.