Necessity of electrically conductive pili for methanogenesis with magnetite stimulation

Oumei Wang Corresp., [1] , **Shiling Zheng** [2] , **Bingchen Wang** [2, 3] , **Wenjing Wang** [2] , **Fanghua Liu** Corresp. [2]

[1] Binzhou Medical University, Yantai, China

[2] Key Laboratory of Coastal Biology and Biological Resources Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences., Yantai, Shandong, China

[3] University of Chinese Academy of Sciences, Beijing, China

Corresponding Authors: Oumei Wang, Fanghua Liu
Email address: omwang@aliyun.com, fhliu@yic.ac.cn

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.

Necessity of electrically conductive pili for methanogenesis with magnetite stimulation

Oumei Wang[1,*], Shiling Zheng[2], Bingchen Wang[2,3], Wenjing Wang[2], Fanghua Liu[2,*]

[1]Binzhou Medical University, Yantai, China; [2]Key Laboratory of Coastal Biology and Biological Resources Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China; [3]University of Chinese Academy of Sciences, Beijing, China.

*Corresponding author

Oumei Wang, Binzhou Medical University, 346 Guanhai Road, Laishan District, Yantai, Shandong 264003, China. Email: omwang@aliyun.com.

or Fanghua Liu, Yantai Institute of Coastal Zone Research, 17 Chunhui Road, Laishan District, Yantai, Shandong 264003, China. Email: fhliu@yic.ac.cn.

Running title: E-pili for magnetite stimulation

ABSTRACT

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.

Subjects Environmental Sciences, Microbiology, Biomineralogy, Ecology

Keywords *Geobacter metallireducens*, *Methanosarcina barkeri*, direct interspecies electron transfer (DIET), electrically conductive pili (e-pili), magnetite, methane, ferrous iron, ethanol metabolism, stimulation, co-cultures

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.

120 Concentrations of ethanol and acetate were analysed with high-performance liquid
121 chromatography (1260 Infinity; Agilent Technologies, USA) with a Hi-plex H column equipped
122 with a refractive index detector.
123 The concentration of HCl-extractable Fe(II) was extracted from cultures and each replicate
124 of the assays in triplicate as described previously (*Zheng et al*., *2015*). Moreover, the
125 concentration of dissolved Fe(II) in samples was also quantified by filtering through 0.45 μm
126 sterile syringe filters and using the ferrozine method as described above.

127 **Microscopy**

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

130 ## RESULTS AND DISCUSSION

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

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

138 The initial establishment of *G. metallireducens* and *M. barkeri* co-cultures requires a long
139 adaption period in the absence of added conductive materials (*Rotaru et al*., *2014a*). As expected,
140 ethanol was only slowly metabolised over 40 days without magnetite (Fig. 1C), however, in the
141 presence of magnetite, ethanol was metabolised with the production of methane beginning within
142 10 days (Fig. 1C). Non-inoculated controls with magnetite showed no ethanol metabolism or
143 methane production.
144 Limited acetate accumulated in the *G. metallireducens* with, or without, *M. barkeri* co-
145 cultures in the presence of magnetite ($C_2H_6O + H_2O \rightarrow C_2H_4O_2 + 4H^+ + 4e^-$), but was later

consumed (Fig. 1D), which differed from co-cultures of *G. metallireducens* and *M. barkeri* in the absence of magnetite, suggesting that *G. metallireducens* metabolised the acetate that *G. metallireducens* produced from ethanol compared with the result of *G. metallireducens* acting alone with magnetite. The high amount of methane in the *G. metallireducens* and *M. barkeri* co-cultures suggested that *M. barkeri* only used the electrons released from ethanol oxidation for reducing carbon dioxide to produce methane in the magnetite-amended co-cultures ($8H^+ + 8e^- + CO_2 \rightarrow CH_4 + 2H_2O$). The total amount of ethanol from the magnetite-amended co-cultures metabolised (1.15 ± 0.12 mmol) resulted in 1.60 ± 0.0032 mmol methane (Figs 1A, C), about 92.2 % of the electrons from ethanol oxidation were recovered in methane according to the equation: $2C_2H_5OH \rightarrow 3CH_4 + CO_2$. Furthermore, no H_2 was detected in any of the experiment groups. This result was consistent with the fact that *G. metallireducens* is unable to produce H_2 during ethanol metabolism (*Shrestha et al*., *2013b*). Therefore, the high electron recovery that was available from ethanol to methane suggested that magnetite can stimulate DIET between *G. metallireducens* and *M. barkeri* and suggested that the simplest explanation for the enrichment of *Geobacter* and *Methanosarcina* observed in the presence of magnetite in previous studies (*Kato et al*., *2012a*) is that magnetite was facilitating DIET.

HCl-extractable ferrous iron was also produced from reduction ferric iron of magnetite within five days and increased to 0.1768 ± 0.0219 mmol at 50 days, which was equal to that 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. 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

173 days in the presence of magnetite, while ferrous iron production from *G. sulfurreducens* amended
174 with magnetite was much lower than that from *G. metallireducens* when utilising acetate (Fig. 2).
175 This suggested that *G. metallireducens*, like some microorganisms (*e.g.*, *Shewanella*,
176 *Dechloromonas*, *Desulfovibrio*, and *Clostridium*) was able to use magnetite as the electron
177 acceptor from ethanol or acetate metabolism (*Kostka & Nealson 1995*; *Yang et al*., *2015*).
178 However, it is not possible for magnetite to act as the electron shuttle for production of methane
179 from carbon dioxide because of the relatively high mid-point potential of the Fe(III)/Fe(II) redox
180 couple (E_0' = +0.20 V, pH 7.0) which is too high for the reduction of carbon dioxide to methane
181 (E_0' of CO_2/methane couple = -240 mV).
182 To determine the actual role of magnetite in stimulation of ethanol metabolism and methane
183 production in co-cultures of wild-type *G. metallireducens* and *M. barkeri*, co-cultures were
184 initiated with 2.5 mmol L^{-1} magnetite, after a 10-day incubation period, an additional 2.5 mmol L^{-1}
185 magnetite was subsequently added. Methane production presented the same tendency with 5
186 mmol L^{-1} magnetite added to the co-cultures (Fig. 1A): this meant that the manner and amount of
187 addition of magnetite could not affect methane production, however, the amount of HCl-
188 extractable ferrous iron changed: magnetite was firstly reduced by *G. metallireducens* and
189 subsequently reduced when more magnetite was added, the amount of ferrous iron used in each
190 step (total: 0.1635 ± 0.0313 mmol) was similar to the addition of 5 mmol L^{-1} magnetite (Fig. 1B).
191 This result was consistent with the observation that *G. metallireducens* alone reduced magnetite
192 to produce ferrous iron. These results further confirmed that magnetite acted as the electron
193 acceptor and could be reduced, to a significant extent, by *G. metallireducens*. Similarly, ethanol
194 was stimulated to oxidise and little acetate was transiently accumulated in magnetite upon its
195 step-by-step addition to co-cultures of wild-type *G. metallireducens* and *M. barkeri* (Figs 1C, D).
196 The calculation of electron recovery (93.81%) of electrons available from ethanol in methane in
197 these samples further suggested that *M. barkeri* was accepting electrons from carbon dioxide
198 reduction via DIET.
199 Transmission electron microscopy (TEM) revealed that *G. metallireducens* and *M. barkeri*

were closely associated (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.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This research was supported by the Major Research Plan (No. 91751112) and the General Programme (No.41371257 and 41573071) of The National Natural Science Foundation of China, the Key Research Project of Frontier Science (No. QYZDJ-SSW-DQC015) of the Chinese Academy of Sciences, the Natural Science Foundation of Shandong Province (Grant no. ZR2016DQ12), and the Young Taishan Scholars Programme (No. tsqn20161054).

Competing interests

The authors declare there are no competing interests.

REFERENCES

253 Adhikari RY, Malvankar NS, Tuominen MT, and Lovley DR. 2016. Conductivity of individual
254 *Geobacter* pili. *Rsc Advances* 6:8363-8366. 10.1039/c5ra28092c
255 Aklujkar M, Krushkal J, DiBartolo G, Lapidus A, Land ML, and Lovley DR. 2009. The genome
256 sequence of *Geobacter metallireducens*: features of metabolism, physiology and regulation
257 common and dissimilar to Geobacter sulfurreducens. *BMC Microbiology* 9. 10.1186/1471-
258 2180-9-109
259 Bagnara C, Toci R, Gaudin C, and Belaich JP. 1985. Isolation and characterization of a
260 cellulolytic microorganism, *cellulomonas fermentans* sp. nov. *International Journal of*
261 *Systematic Bacteriology* 35:502-507.
262 Bridgham SD, Cadillo-Quiroz H, Keller JK, and Zhuang Q. 2013. Methane emissions from
263 wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales.
264 *Global Change Biology* 19:1325-1346. 10.1111/gcb.12131
265 Chen S, Rotaru A-E, Liu F, Philips J, Woodard TL, Nevin KP, and Lovley DR. 2014a. Carbon
266 cloth stimulates direct interspecies electron transfer in syntrophic co-cultures. *Bioresource*
267 *technology* 173:82-86. 10.1016/j.biortech.2014.09.009
268 Chen S, Rotaru A-E, Shrestha PM, Malvankar NS, Liu F, Fan W, Nevin KP, and Lovley DR.
269 2014b. Promoting interspecies electron transfer with biochar. *Scientific Reports* 4.
270 10.1038/srep05019
271 Conrad R. 2007. Microbial ecology of methanogens and methanotrophs. In: Sparks DL, ed.
272 *Advances in Agronomy, Vol 96*, 1-63.
273 Kang YS, Risbud S, Rabolt JF, and Stroeve P. 1996. Synthesis and characterization of nanometer-
274 size Fe_3O_4 and γ-Fe_2O_3 particles. *Chemistry of Materials* 8:2209-2211. 10.1021/cm960157j
275 Kato S, Hashimoto K, and Watanabe K. 2012a. Methanogenesis facilitated by electric syntrophy
276 via (semi) conductive iron-oxide minerals. *Environmental Microbiology* 14:1646-1654.
277 10.1111/j.1462-2920.2011.02611.x
278 Kato S, Hashimoto K, and Watanabe K. 2012b. Microbial interspecies electron transfer via
279 electric currents through conductive minerals. *Proceedings of the National Academy of*
280 *Sciences of the United States of America* 109:10042-10046. 10.1073/pnas.1117592109

281 Kostka JE, and Nealson KH. 1995. Dissolution and reduction of magnetite by bacteria.
282 *Environmental Science & Technology* 29:2535-2540. 10.1021/es00010a012
283 Leang C, Qian X, Mester T, and Lovley DR. 2010. Alignment of the c-type cytochrome OmcS
284 along pili of *Geobacter sulfurreducens*. *Applied and Environmental Microbiology* 76:4080-
285 4084. 10.1128/aem.00023-10
286 Li H, Chang J, Liu P, Fu L, Ding D, and Lu Y. 2015. Direct interspecies electron transfer
287 accelerates syntrophic oxidation of butyrate in paddy soil enrichments. *Environmental*
288 *Microbiology* 17:1533-1547. 10.1111/1462-2920.12576
289 Liu F, Rotaru A-E, Shrestha PM, Malvankar NS, Nevin KP, and Lovley DR. 2012. Promoting
290 direct interspecies electron transfer with activated carbon. *Energy & Environmental Science*
291 5:8982-8989. 10.1039/c2ee22459c
292 Liu F, Rotaru A-E, Shrestha PM, Malvankar NS, Nevin KP, and Lovley DR. 2015. Magnetite
293 compensates for the lack of a pilin-associated *c*-type cytochrome in extracellular electron
294 exchange. *Environmental Microbiology* 17:648-655. 10.1111/1462-2920.12485
295 Liu FH, Wang SB, Zhang JS, Zhang J, Yan X, Zhou HK, Zhao GP, Zhou ZH. 2009. The structure
296 of the bacterial and archaeal community in a biogas digester as revealed by denaturing
297 gradient gel electrophoresis and 16S rDNA sequencing analysis. *Journal of Applied*
298 *Microbiology* 106:952-966. 10.1111/j.1365-2672.2008.04064.x
299 Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJP, Gorby YA, and Goodwin S.
300 1993. *Geobacter metallireducens* gen. nov. sp. nov, a microorganism capable of coupling the
301 complete oxidation of organic-compounds to the reduction of iron and other metals. *Archives*
302 *of Microbiology* 159:336-344. 10.1007/bf00290916
303 Malvankar NS, and Lovley DR. 2014. Microbial nanowires for bioenergy applications. *Current*
304 *Opinion in Biotechnology* 27:88-95. 10.1016/j.copbio.2013.12.003
305 Malvankar NS, Tuominen MT, and Lovley DR. 2012. Lack of cytochrome involvement in long-
306 range electron transport through conductive biofilms and nanowires of *Geobacter*
307 *sulfurreducens*. *Energy & Environmental Science* 5:8651-8659. 10.1039/c2ee22330a
308 Malvankar NS, Vargas M, Nevin K, Tremblay P-L, Evans-Lutterodt K, Nykypanchuk D, Martz

309 E, Tuominen MT, and Lovley DR. 2015. Structural basis for metallic-like conductivity in
310 microbial nanowires. *Mbio* 6. 10.1128/mBio.00084-15
311 Malvankar NS, Vargas M, Nevin KP, Franks AE, Leang C, Kim B-C, Inoue K, Mester T, Covalla
312 SF, Johnson JP, Rotello VM, Tuominen MT, and Lovley DR. 2011. Tunable metallic-like
313 conductivity in microbial nanowire networks. *Nature Nanotechnology* 6:573-579.
314 10.1038/nnano.2011.119
315 Mehta T, Coppi MV, Childers SE, and Lovley DR. 2005. Outer membrane *c*-type cytochromes
316 required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Applied and*
317 *Environmental Microbiology* 71:8634-8641. 10.1128/aem.71.12.8634-8641.2005
318 Rotaru A-E, Shrestha PM, Liu F, Markovaite B, Chen S, Nevin K, and Lovley D. 2014a. Direct
319 interspecies electron transfer between *Geobacter metallireducens* and *Methanosarcina*
320 *barkeri*. *Applied and Environmental Microbiology*:4599-4605. 10.1128/aem.00895-14
321 Rotaru A-E, Shrestha PM, Liu F, Shrestha M, Shrestha D, Embree M, Zengler K, Wardman C,
322 Nevin KP, and Lovley DR. 2014b. A new model for electron flow during anaerobic
323 digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon
324 dioxide to methane. *Energy & Environmental Science* 7:408-415. 10.1039/c3ee42189a
325 Shen L, ZhaoQC, Wu X, Li XZ, Li QB, Wang YP. 2016. Interspecies electron transfer in
326 syntrophic methanogenic consortia: From cultures to bioreactors. *Renewable & Sustainable*
327 *Energy Reviews* 54:1358-1367. 10.1016/j.rser.2015.10.102
328 Shrestha PM, Kube M, Reinhardt R, and Liesack W. 2009. Transcriptional activity of paddy soil
329 bacterial communities. *Environmental Microbiology* 11:960-970. 10.1111/j.1462-
330 2920.2008.01821.x
331 Shrestha PM, Rotaru A-E, Summers ZM, Shrestha M, Liu F, and Lovley DR. 2013a.
332 Transcriptomic and genetic analysis of direct interspecies electron transfer. *Applied and*
333 *Environmental Microbiology* 79:2397-2404. 10.1128/aem.03837-12
334 Shrestha PM, Rotaru AE, Aklujkar M, Liu FH, Shrestha M, Summers ZM, Malvankar N, Flores
335 DC, and Lovley DR. 2013b. Syntrophic growth with direct interspecies electron transfer as
336 the primary mechanism for energy exchange. *Environmental Microbiology Reports* 5:904-

910. 10.1111/1758-2229.12093

Sieber JR, McInerney MJ, and Gunsalus RP. 2012. Genomic insights into syntrophy: The paradigm for anaerobic metabolic cooperation. In: Gottesman S, Harwood CS, and Schneewind O, eds. *Annual Review of Microbiology, Vol 66*, 429-452.

Smith JA, Lovley DR, and Tremblay P-L. 2013. Outer cell surface components essential for Fe(III) oxide reduction by *Geobacter metallireducens*. *Applied and Environmental Microbiology* 79:901-907. 10.1128/aem.02954-12

Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, and Lovley DR. 2010. Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* 330:1413-1415. 10.1126/science.1196526

Tang J, Zhuang L, Ma J, Tang Z, Yu Z, and Zhou S. 2016. Secondary mineralization of ferrihydrite affects microbial methanogenesis in *Geobacter-Methanosarcina* cocultures. *Applied and Environmental Microbiology* 82:5869-5877.

Tremblay P-L, Aklujkar M, Leang C, Nevin KP, and Lovley D. 2012. A genetic system for *Geobacter metallireducens*: role of the flagellin and pilin in the reduction of Fe(III) oxide. *Environmental Microbiology Reports* 4:82-88. 10.1111/j.1758-2229.2011.00305.x

Vargas M, Malvankar NS, Tremblay P-L, Leang C, Smith JA, Patel P, Synoeyenbos-West O, Nevin KP, and Lovley DR. 2013. Aromatic amino acids required for pili conductivity and long-range extracellular electron transport in *Geobacter sulfurreducens*. *Mbio* 4. 10.1128/mBio.00105-13

Viggi CC, Rossetti S, Fazi S, Paiano P, Majone M, and Aulenta F. 2014. Magnetite particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation. *Environmental Science & Technology* 48:7536-7543. 10.1021/es5016789

Wang L-Y, Nevin KP, Woodard TL, Mu B-Z, and Lovley DR. 2016. Expanding the diet for DIET: Electron donors supporting direct interspecies electron transfer (DIET) in defined co-cultures. *Frontiers in Microbiology* 7. 10.3389/fmicb.2016.00236

Yang ZM, Shi XS, Wang CS, Wang L, and Guo RB. 2015. Magnetite nanoparticles facilitate methane production from ethanol via acting as electron acceptors. *Scientific Reports* 5.

10.1038/srep16118

Zheng S, Liu F, Li M, Xiao L, and Wang O. 2017. Comparative transcriptomic insights into the mechanisms of electron transfer in *Geobacter* co-cultures with activated carbon and magnetite. *Science China Life Sciences*. 10.1007/s11427-017-9177-1

Zheng S, Zhang H, Li Y, Zhang H, Wang O, Zhang J, and Liu F. 2015. Co-occurrence of *Methanosarcina mazei* and *Geobacteraceae* in an iron (III)-reducing enrichment culture. *Frontiers in Microbiology* 6. 10.3389/fmicb.2015.00941

Zhuang L, Xu JL, Tang J, and Zhou SG. 2015. Effect of ferrihydrite biomineralization on methanogenesis in an anaerobic incubation from paddy soil. *Journal of Geophysical Research-Biogeosciences* 120:876-886. 10.1002/2014jg002893

Figures

Figure 1. 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. 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.

Figure 3. Transmission electron micrographs. Association of the defined co-cultures of *G. metallireducens* and *M. barkeri* with magnetite. (A) Association of the two cell types. (B) Association of magnetite with pili. Black arrows and white pentagrams indicate *G. metallireducens* cells and *M. barkeri* cells, respectively. White arrows indicate magnetite and pili.

Figure 4. 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

391 the symbol.

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.

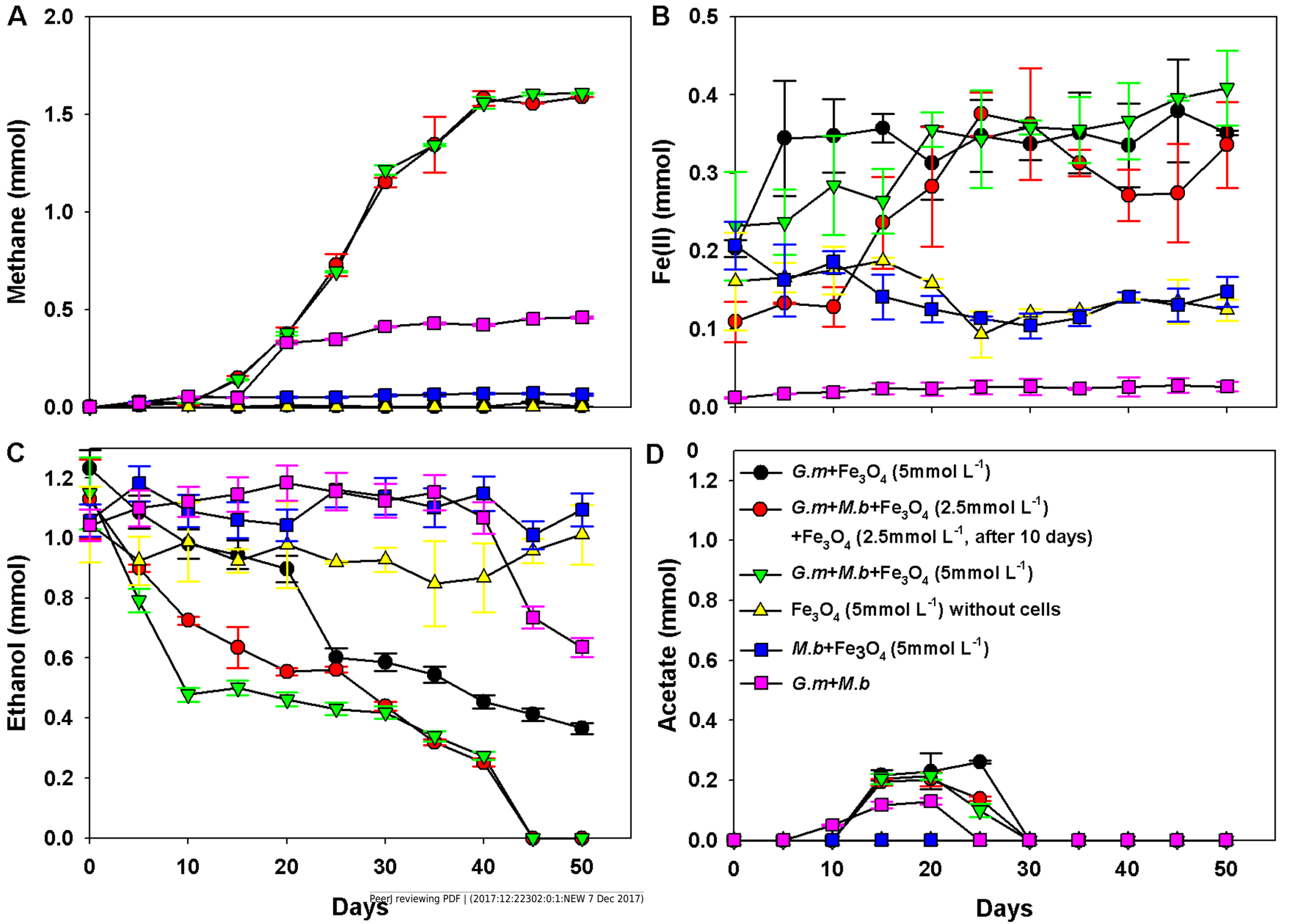

A
Methane (mmol)
2.0
1.5
1.0
0.5
0.0
B
Fe(II) (mmol)
0.5
0.4
0.3
0.2
0.1
0.0
C
Ethanol (mmol)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Days
D
Acetate (mmol)
0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Days
G.m+Fe3O4 (5mmol L-1)
G.m+M.b+Fe3O4 (2.5mmol L-1)
+Fe3O4 (2.5mmol L-1, after 10 days)
G.m+M.b+Fe3O4 (5mmol L-1)
Fe3O4 (5mmol L-1) without cells
M.b+Fe3O4 (5mmol L-1)
G.m+M.b

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.

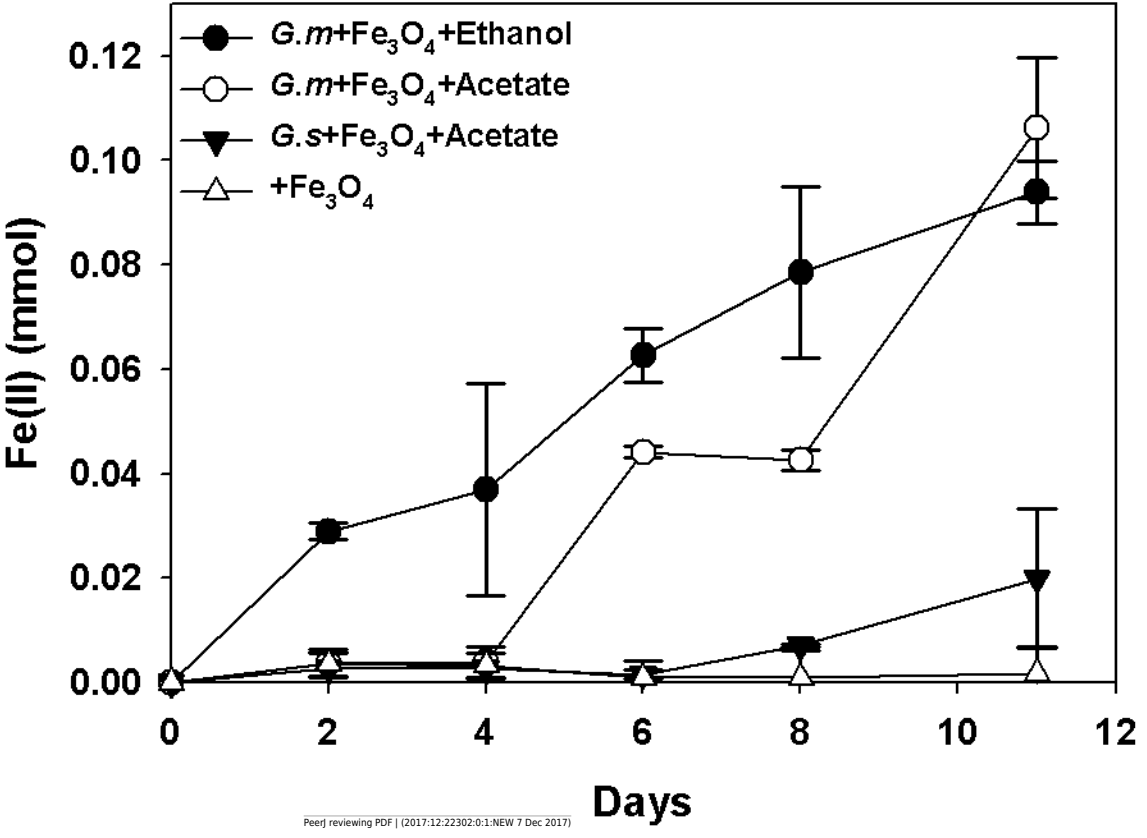

G.m+Fe3O4+Ethanol
G.m+Fe3O4+Acetate
G.s+Fe3O4+Acetate
+Fe3O4
Fe(II) (mmol)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0
2
4
6
8
10
12
Days

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. (B) Association of magnetite with pili. Black arrows and white pentagrams indicate *G. metallireducens* cells and *M. barkeri* cells, respectively. White arrows indicate magnetite and pili.

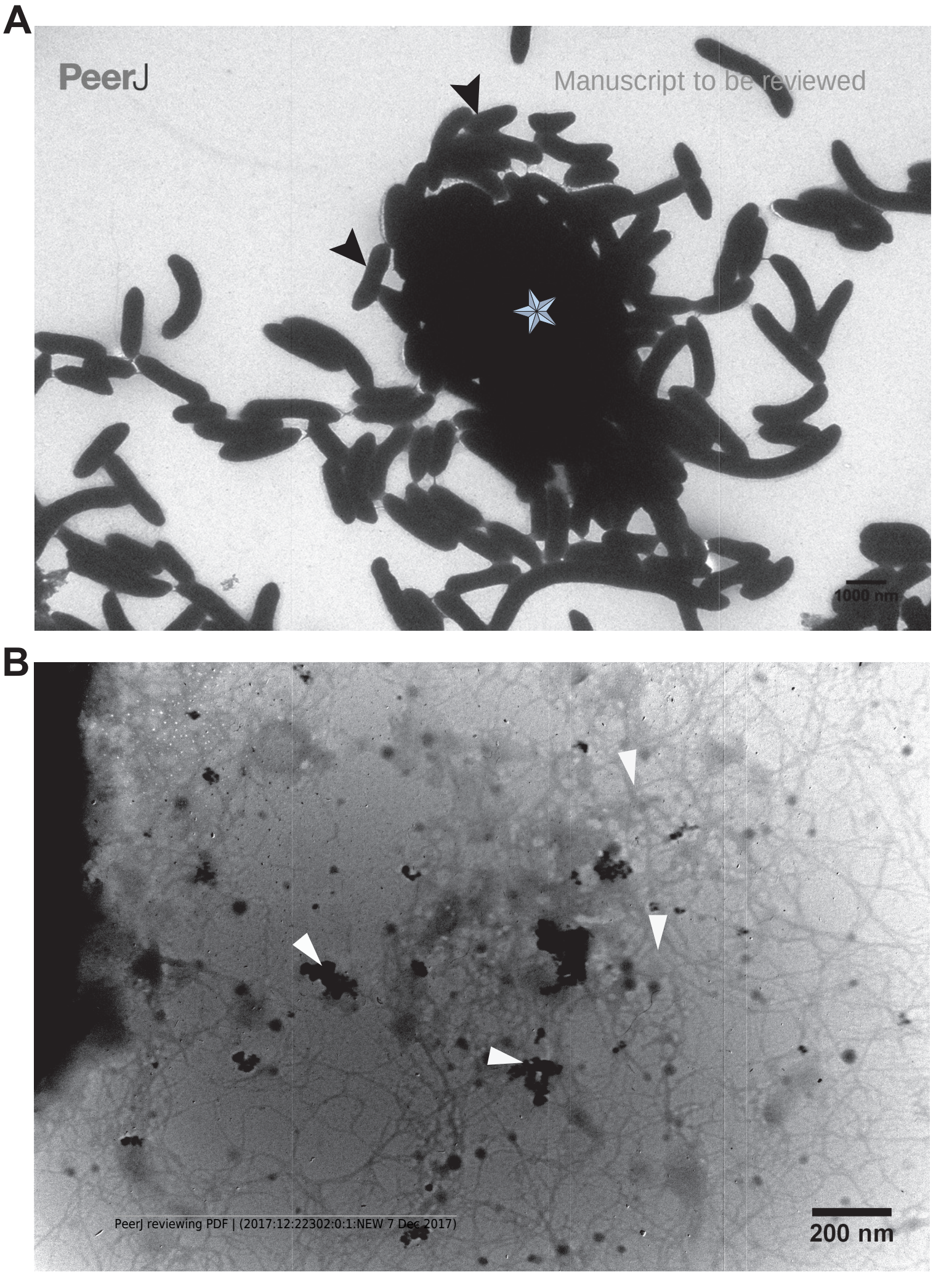
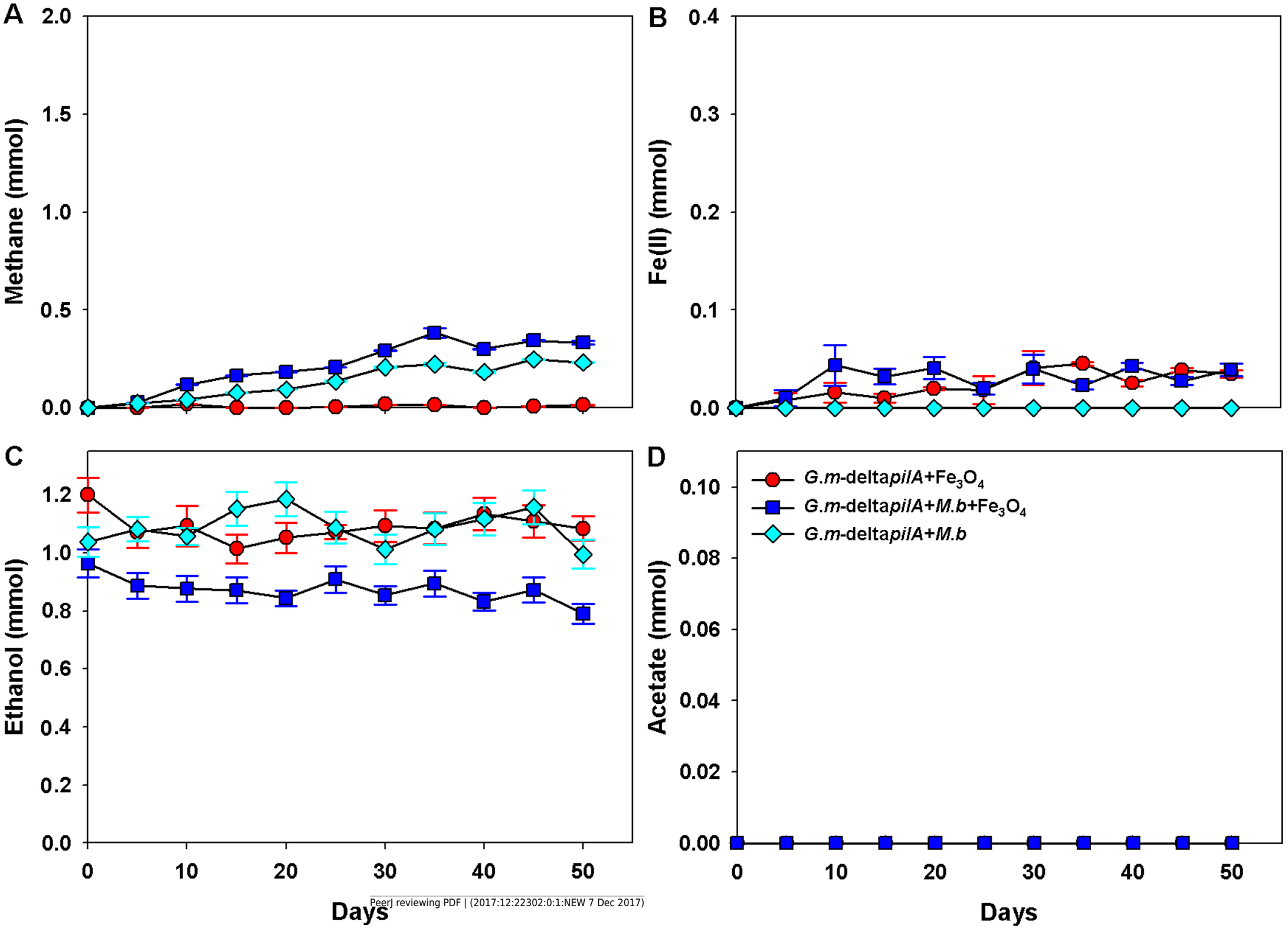


Figure 4(on next page)

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.


A
Methane (mmol)
2.0
1.5
1.0
0.5
0.0
B
Fe(II) (mmol)
0.4
0.3
0.2
0.1
0.0
C
Ethanol (mmol)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Days
D
Acetate (mmol)
0.10
0.08
0.06
0.04
0.02
0.00
G.m-deltapilA+Fe3O4
G.m-deltapilA+M.b+Fe3O4
G.m-deltapilA+M.b
0
10
20
30
40
50
Days