1   The impact of invader number on whole community invasions in biomethane-producing

2   communities

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16   **Abstract**

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18   Microbes can invade as whole communities, but the ecology of whole community invasions are

19   poorly understood. Here, we investigate how invader frequency affects the composition and

20   function of invaded laboratory methanogenic communities. An invading community was equally

21   successful at establishing itself in a resident community regardless of initial invader frequency,

22   which varied between 0.01 and 10%. Invasion resulted in enhanced biogas production (to the

23   level of the pure invading community), but only when invader frequency was 1% or greater.

24   This inconsistency between invasion success and changes in function can be explained by a

25   lower number of invading taxa (but not individuals) at lower initial invader frequencies, and an

26   important functional role of the taxa that were absent. Our results highlight that whole

27   community invasion ecology cannot simply be extrapolated from our understanding of single

28   species invasions. Moreover, we show that methane production can be enhanced by invading

29   poorly performing reactors with a better performing community at levels that may be practical

30   in industrial settings.

31

32   **Introduction**

33

34   Plant and animal invaders can play a major role in the structure and function of natural

35   ecosystems [1–3]. Microbial populations and communities, like those of plants and animals, are

36   also geographically structured [4–7], suggesting an important role of microbial invasions in their

37   formation. We know relatively little about the cause and consequences of microbial invasions

38   [8, 9], but there are clear parallels with the more extensive research on plant and animal

39   invasion ecology [10–13]. In microbes for example, resident diversity typically reduces invasion

40   success [14, 15], while invader population sizes [16–18] and disturbances [19, 20] may promote

41   invasion. However, there are also likely to be major differences between microbial and

42   macrobial invader dynamics. Notably, microbes often invade as whole communities, rather than

43   as single species [21]. Leaves falling on the ground [22] or the release of sewage into the rivers

44   [23] are examples of entire microbial assemblies arriving in a new ecosystem.

45

46   Recent theoretical and empirical work has started to identify conditions that affect the likelihood

47   of whole community invasions [24, 25]. Resident diversity and productivity inhibit invasion [26,

48   27], while increasing invader diversity boosts invasion success (Rivett *et al.* 2018; Vila *et al.*

49   2019). Here, we focus on another theoretically important variable for both invading communities

50   and populations: The number of invaders [17, 18, 28], or propagule size. A higher number of

51   invaders can increase invasion success for a range of reasons. First, by reducing stochastic

52   loss of invaders. Second, community size is likely to positively correlate with its species diversity

53   [29, 30], meaning that a larger invading community is both more likely to contain a particularly

54   invasive species, and show greater niche complementarity. The latter increases positive co-

55   selection between community members [20, 26, 27]. Third, larger population sizes of invading

56   species are also likely to result in invader populations adapting to new ecological conditions

57   more rapidly [31].

58

59   While the number of invaders is expected to increase invasion success of single species and

60   whole communities, the functional consequences of invasion may be less predictable for whole

61   communities. Natural microbial communities are typically very diverse, meaning that stochastic

62   loss of individual species within communities is increasingly likely with lower numbers of

63   invaders. The rare taxa are more likely to be lost and there is growing evidence that rare taxa

64   play a crucial role in community functions [20, 32, 33]. This means that invasions by lower

65   numbers of invaders, even when successful, are less likely to be linked to key functional

66   changes in the community than a similarly successful invasion initiated by a higher number of

67   individuals. By contrast, a successful invasion by a single species might be expected to have

68   similar consequences for community function regardless of initial number of invaders.

69

70   Here, we empirically investigate the relationships between invader numbers, invasion success,

71   and functional consequences in methanogenic communities. Methane is an end product of

72   community metabolism in these communities and therefore a useful proxy of community

73   resource use efficiency (productivity). We use methanogenic communities because of their

74   relevance to both biotechnology, where methane is playing an increasingly important role in the

75   world's renewable energy portfolio, and climate change in general, where melting permafrost

76   may increase community mixing rates and impact methane release to the atmosphere [34].

77   Importantly, successful invasions are likely to result in increased methane production by the

78   resultant community, as the higher methane producing communities have been shown to

79   dominate when multiple communities are mixed together [27].

80

81   **Materials and Methods**

82

83   Experimental design

84   We used communities isolated from two anaerobic digester (AD) plants from the UK South

85   West region. Before the experiment, we ran a pilot experiment for 4 weeks to measure methane

86   production of each community. The stock communities were stored at 4°C for the duration of

87   the experiment. We selected the community that produced the least methane of the two (Low

88   Producer, LP) and cultivated 25 LP replicate fermenters using this community and 5 replicate

89   fermenters of the community that produced more methane (High Producer; HP) in fermenters

90   for two weeks to allow them to pre-adapt to experimental conditions. After two weeks, we

91   invaded 20 of the LP replicates with either 10%, 1%, 0.1%, and 0.01% (by volume) of one of

92   the HP replicates (5 replicates for each invasion percentage), while 5 LP replicates and 5 HP

93   replicates were left undisturbed. The invaded LP communities and pure LP and HP

94   communities were cultivated for an additional 6 weeks post-invasion, with gas production

95   measured throughout and community composition determined at the beginning and end of the

96   experiment.

97

98   Cultivation and gas measurement

99   All replicates were grown in 500 mL bottles (Duran, 600mL total volume with headspace) using

100   Automated Methane Potential Test System (AMPTS, Bioprocess Control Sweden AB)[27] to

101   measure CO_2-stripped biogas (Biogas) production. The pilot experiment and the pure LP and

102   HP communities were started with 200 mL of the community. The 10% Invasion replicates were

103   initiated with 180 mL of LP community and invaded with 20 mL of HP community after 2 weeks,

104   while 1%, 0.1%, and 0.01% treatments were initiated with 198 mL of LP and invaded with 2 mL

105   of either pure or HP, or HP diluted 10 or 100-fold with ddH$_2$O, as appropriate. Each community

106   was supplemented with 0.2 mL 1000x concentrated trace metal solution (1 g L^{-1} FeCl$_2$ · 4H$_2$O,

107   0.5 g L^{-1} MnCl$_2$ · 4H$_2$O, 0.3 g L^{-1} CoCl$_2$ · 4H$_2$O, 0.2 g L^{-1} ZnCl$_2$, 0.1 g L^{-1} NiSO$_4$ · 6H$_2$O, 0.05 g

108   L^{-1} Na$_2$MoO$_4$ · 4H$_2$O, 0.02 g L^{-1} H$_3$BO$_3$, 0.008 g L^{-1} Na$_2$WO$_4$ · 2H$_2$O, 0.006 g L^{-1} Na$_2$SeO$_3$ · 5H$_2$O,

109   0.002 g L^{-1} CuCl$_2$ · 2H$_2$O) at the start of the experiment. In both the pilot and main experiments,

110   all communities were fed weekly, starting on t_0 with 2g of feed composed of 3.53% casein,

111   1.17% peptone, 1.17% albumen, 47.07% dextrin, and 47.07% sucrose (all compounds –

112   Sigma), The feed was suspended in 18 mL sterile water.

113

114   DNA extractions and sequencing

115   DNA was extracted and sequenced as described previously [33]. We extracted DNA using

116   FastDNA™ SPIN Kit for Soil (MP). We confirmed the quality of extractions by electrophoresis

117   on a 1% agarose gel, and we quantified it using dsDNA BR (Qubit). We constructed the 16S

118   rRNA gene libraries using primers designed to amplify the V4 region [35] (Supplementary Table

119   S1) and multiplexed. We generated amplicons using a high-fidelity polymerase (Kapa 2G

120   Robust) and purified them using the Agencourt AMPure XP PCR purification system. They

121   were quantified fluorometrically (Qubit, Life Technologies). We pooled the amplicons in

122   equimolar concentrations based on Qubit results. We diluted the amplicon library pool to 2 nM

123   in sodium hydroxide and transferred 5 µL into 995 µL HT1 (Illumina) to give a final concentration

124   of 10 pM. We spiked 600 µL of the diluted library pool with 10% PhiX Control v3 on ice before

125   loading into the Illumina MiSeq cartridge following the manufacturer's instructions. The

126   sequencing chemistry utilised was MiSeq Reagent Kit v2 (500 cycles) with run metrics of 250

127   cycles for each paired-end read using MiSeq Control Software 2.2.0 and RTA 1.17.28. One of

128   the samples from HP treatment failed to sequence and is not included in the analysis.

129

130   Data analysis

131   Sequencing data were analysed in R (v 3.3.2) using the packages *'dada2'* [36], *'phyloseq'* [37],

132   *'vegan'* [38] and *'pairwiseAdonis'* [39]. We used a full-stack workflow to estimate error rates,

133   inferred and merged sequences, constructed a sequence table, removed chimeric sequences,

134   and assigned taxonomy to each amplicon sequence variant (ASV) using the Greengenes

135   database [40] following the published pipeline [36]. We estimated the phylogenetic tree using

136   the R package *'phangorn'*, [41] where we constructed a neighbour-joining tree, and then fit a

137   Generalized time-reversible with Gamma rate variation maximum likelihood tree using the

138   neighbour-joining tree as a starting point. We used UniFrac distance (weighted and

139   unweighted) as our measure of compositional dissimilarity between the communities

140   (Lozupone & Knight 2005) . In case of alpha diversity, we calculated species richness and

141   evenness [43].

142

143   To establish if the invasions were successful, we needed to determine if organisms from the

144   HP treatment managed to establish themselves in the invasion treatment. The majority of ASVs

145   were shared between HP and LP samples (Fig. 1) making it difficult to discriminate relative

146   community contributions using previous statistical methods [27]. Instead we subset the 16S

147   sequencing data. For each invaded community, we determined the ASVs of attributable origin:

148   those that were present in the endpoint of only one of the pure community treatments. ASVs

149   found in LP community, but not in HP were attributed to LP and vice versa. ASVs present in

150   both or neither pure community treatments were excluded. Invasion success was then

151   estimated in two ways: as the proportion of ASV reads of HP origin in total reads (proxy for the

152   fraction of successfully invaded individuals) or as a proportion of ASVs of HP origin in all ASVs

153   (proxy for fraction of species that successfully invaded the community). Separate linear models

154   were used to determine whether final invader number or invader diversity was altered by initial

155   invader abundance, where invader abundance was treated as a categorical predictor. Model

156   selection was done using likelihood ratio tests and post-hoc pairwise comparisons were

157   between individual treatments.

158

159   When looking at different community composition between treatments and pure LP and HP

160   communities, we used all of the 16S sequencing data including all ASVs present in single

161   treatments and pure LP and HP communities. We used a single permutational ANOVA to look

162   at an overall difference in community composition between treatments, and used pairwise

163   permutational ANOVA to see which treatments were driving any observed pattern. Differences

164   in alpha diversity and evenness were tested using linear models, with number of ASVs or

165   Pielou's evenness as the response variable and treatment (invasion density or pure LP/HP

166   community) as the predictor variable. Model selection and post-hoc comparisons were done as

167   above. Similarly, gas production was compared using linear models, with gas production as a

168   response variable and treatment (invasion density or pure LP/HP community) as the predictor

169   variable. We used Dunnett's Test to compare the invasion treatments to pure LP or pure HP

170   gas production only, thus avoiding multiple comparisons between the invasion treatments.

171   Model selection and post-hoc comparisons were done as above.

172

173   **Results**

174

175   Invasion Success

176   While only between 4 and 9% of all reads and between 40 and 47% of all ASVs in the invaded

177   communities were attributable to only 1 community, the HP community appeared to

178   successfully invade the LP community in all cases. The mean percentage of invading reads in

179   total reads of attributable origin (Fig. 2A) varied between 8.5% and 60.3%. The mean

180   percentage of invader ASVs in total ASVs of attributable origin (Fig. 2B) was between 16.3%

181   and 46.6%. This means that even if all the non-attributed reads and ASVs originated from the

182   LP community, as much as 0.7% and 7%, for total reads and numbers of ASVs, respectively,

183   would still be attributable to the invading community (HP), much higher fraction than the lowest

184   invader input of 0.01%. While the percentage of HP reads did not vary with the initial inoculum

185   size of the invading community (Fig. 2B, $F_{1,18} = 1.85$, $P = 0.19$), the percentage of HP taxa was

186   higher when initial inoculum size of the invading community was higher ($F_{1,18} = 88.2$, $P < 0.001$).

187   There was an average of 90 invader ASVs in the 10% treatment, this number reduced to 53

188   ASVs in 1% treatment and 30 ASVs in 0.1% and 0.01% treatments.

189

190   Compositional changes following invasion

191   The composition of most of the invaded communities resembled the LP community when

192   considering the abundances of all ASVs found in the samples (Weighted UniFrac): only the

193   10% (and HP) treatments differed in composition from the LP community (Fig. 3A, adonis2,

194    $F_{5,23}$ = 14.59 R^2 = 0.76, P = 0.001, Pairwise adonis: P < 0.01). By contrast, all invaded

195   communities were different from the LP community based on ASV presence and absence only

196   (Unweighted UniFrac) (Fig. 3B, adonis2, $F_{5,23}$ = 7.76, R^2 = 0.62, P < 0.001. Pairwise adonis: P

197   = 0.002). Note that communities changed considerably during propagation, with large

198   differences in composition (using both metrics) largely because of ASV losses.

199

200   We also determined how invasion affected total within- community diversity. The HP community

201   had significantly higher ASV richness than either the LP community or any of the invaded

202   communities (Fig. 4A ANOVA, $F_{5,23}$ = 24.5, P < 0.001, Tukey-Kramer HSD: P < 0.01), and there

203   was a positive relationship between the richness of the invaded LP communities and the dose

204   used in the invasion ($F_{1,27}$ = 43.16, P < 0.001, R^2 = 0.6). Species evenness (Pielou index) did

205   not differ between the treatments (Fig. 4B, ANOVA, $F_{5,23}$ = 0.3, P = 0.91).

206

207   Gas production

208   Total gas production of LP communities increased with the log10 of invader inoculum size (Fig.

209   5B, $F_{1,23}$ = 10.23, P = 0.003 R^2 = 0.28). This enhancement of gas production resulted in the

210   10% and 1% invasion treatments showing gas production no different to the HP treatment,

211   while the 0.1% and 0.01% invasion dose treatments produced significantly less biogas than HP

212   (Fig. 5A, Dunnett Test with HP as control, P < 0.05 for significant differences) and conversely,

213   10% and 1% invasion treatments produce more gas than LP, while 0.1% and 0.01% treatments

214   are no different from it (Fig. 5A, Dunnett Test with LP as control, P < 0.05 for significant

215   differences)

216   Species analysis

217   The communities were composed mainly of *Firmicutes* and *Bacteroidetes* phyla, with methane

218   production driven by primarily acetoclastic methanogens in both communities. Given the

219   apparent "tipping point" in gas production between 0.1 and 1% invasion, we determined if we

220   could correlate this increase in function with particular taxa. We identified 44 ASVs

221   (Supplemental material) that were present in the 10% and 1% but not the 0.1% or 0.01%

222   treatment, and further refined these to 13 ASVs that were detectable in >50% of the 10% and

223   1% replicates. These 13 ASVs were mainly in the *Firmicutes* phylum, with species belonging

224   to the *Ruminococcaceae* family being the most represented (4 ASVs). Aside from the

225   Firmicutes, we found 2 abundant ASVs in the *Bacteroidetes* phylum from the

226   *Porphyromonadaceae* family. One of those, a member of *Petrimonas* genus, was particularly

227   numerous.

228

229   **Discussion**

230

231   We investigated how the outcome of invasion was affected by the number of invading

232   organisms in methanogenic communities. In contrast to our expectation, we found invasion

233   success to be independent of the quantity of the invader used. Biogas production (a proxy for

234   community productivity), was greater in the invaded communities (compared to the LP

235   community), and increased with invader frequency. The relationship was stepped: gas

236   production of the resident community was not altered by 0.01% and 0.1% invasion but

237   resembled that of the invading community with 1 and 10% invader frequencies.

238

239   The disparity between the different measures of invasion success (number of individuals and

240   number of taxa) is presumably because smaller number of invaders results in the loss of rare

241   taxa. Either fewer taxa were initially inoculated into the resident community, or the reduced

242   population size of some taxa resulted in stochastic loss during invasion. In contrast to recent

243   work using synthetic communities [44], rare taxa did not appear to have contributed to the

244   growth of the community as a consequence of co-selection between community members.

245

246   The loss of rare taxa with lower initial invader frequencies can also explain patterns of gas

247   production. Increasing the volume of invading inoculum from 0.1 to 1%, led to approximately

248   40 more ASVs successfully invading and a marked increase in gas production. This is

249   consistent with previous findings that the reduction in number of rare taxa through culture

250   dilution reduced biogas production [33]. Note that the increase in gas production with invader

251   frequency can't be explained by key resources carried over from the media of the invading

252   community, because there was a lag of approximately 4 weeks before gas production in these

253   treatments started to increase.

254

255   While this work demonstrates that communities are capable of invading other communities from

256   very rare, invading communities did not dominate the final community structure. Both theory

257   and our previous experiments using methanogenic communities suggest that the most

258   productive communities will dominate within a mixture of communities. However, in our

259   previous study, unlike here, the communities were mixed at equal frequencies. The lack of

260   invader dominating in low invasion treatments cannot be explained by invaders simply not

261   having reached equilibrium conditions. The proportion of invader reads was the same

262   regardless of invasion treatment, which means the communities stabilised before the endpoint

263   of the experiment. This suggests there may have been a resident advantage through positive

264   frequency or density dependence (a priority effect; e.g. see [45, 46]). This might arise because

265   mutualistic interactions between residents are less disrupted when other community members

266   are relatively rare.

267

268   Given the stepped increase in gas production with invader frequency, the taxa responsible for

269   the increased gas production were presumably those present in the 10% and 1%, but not the

270   0.1% and 0.01%, invader treatments. Thirteen ASVs fitted that criterion and were present in

271   more than half of the 10% and 1% invasion replicates. Only around seven of them could be

272   identified to the family level. Four of the taxa present in majority of the treatments belonged to

273   *Ruminococcaceae, two to Porphyromonadaceae* and one to *Syntrophomonadaceae* families,

274 and are most likely involved in hydrolysis, protein metabolism and direct interspecies electron

275 transfer [47–50]. While the first is not surprising, as the medium used in experiments is

276 predominantly made from complex sugars, the other two point to interesting possibilities.

277 Selection for organisms efficient at protein metabolism functions suggest a link between the

278 increase of the community function and nutrient scavenging. The abundance of *Petrimonas*

279 ASV from *Porphyromonadaceae* family could be linked to facilitating electron transfer between

280 species and use of acetate in biogas production [50]. Given the dominance of acetoclastic

281 methanogens in our bioreactors, this particular ASV is the most likely driver of increased biogas

282 yield in the invaded communities

283

284 What is the relevance of our work to natural communities? Generalisations may be limited given

285 that our laboratory conditions likely promoted invasion: Our communities were continually

286 mixed [51] and experienced novel environments. Natural communities in stable environments

287 are likely to be more locally adapted, promoting a resident advantage [46], and hence invasions

288 will likely play a less important role in changes in community composition [52]. That said, our

289 results are likely most relevant to communities experiencing environmental change, where an

290 invading community is potentially better adapted to at least some local conditions than are the

291 residents. An important implication is that while invasion may readily lead to restoration of a

292 locally adapted community, this may not result in a concomitant restoration of function.

293 methanogenic communities, which show much metabolic interdependence, are particularly

294 prone to whole community invasions compared with other types of communities, such as soil

295 [53], is unclear.

296

297 Our results suggest a potential novel approach to enhancing methane production in industrial

298 settings, although further work is needed. We have previously established that seeding an

299 Anaerobic Digester from a mixture of starting communities will, on average, result in improved

300 gas production because the most productive communities tend to dominate. However,

301 improving performance of existing reactors inevitably requires successful invasion by a smaller

302 community than the resident. We show that invader frequencies as low as 0.01% can

303   successfully invade. While the lower initial invader frequencies did not result in increased gas

304   production, increasing absolute size of invader communities or (as would be the case when

305   scaling up to industrial reactors) could overcome the problem associated with loss of rare taxa.

306

307   **Acknowldgements**

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311   **Competing Interests**

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315   **References**

316

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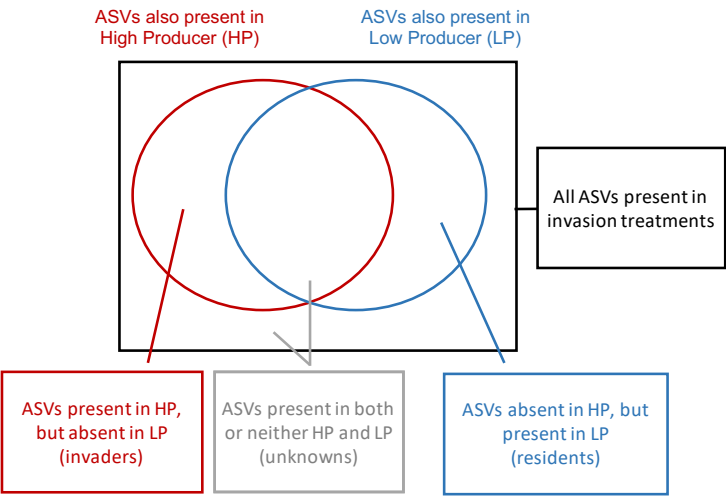
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Figures and Tables

Figure 1: Schematic demonstrating how we attributed reads and ASVs to look at invader density and diversity. We checked if the ASVs found in the invasion treatments were also present in the LP and HP pure communities. ASVs found in one of the pure communities were attributed as originating from that pure community. ASVs found in both or neither pure communities were not attributed.
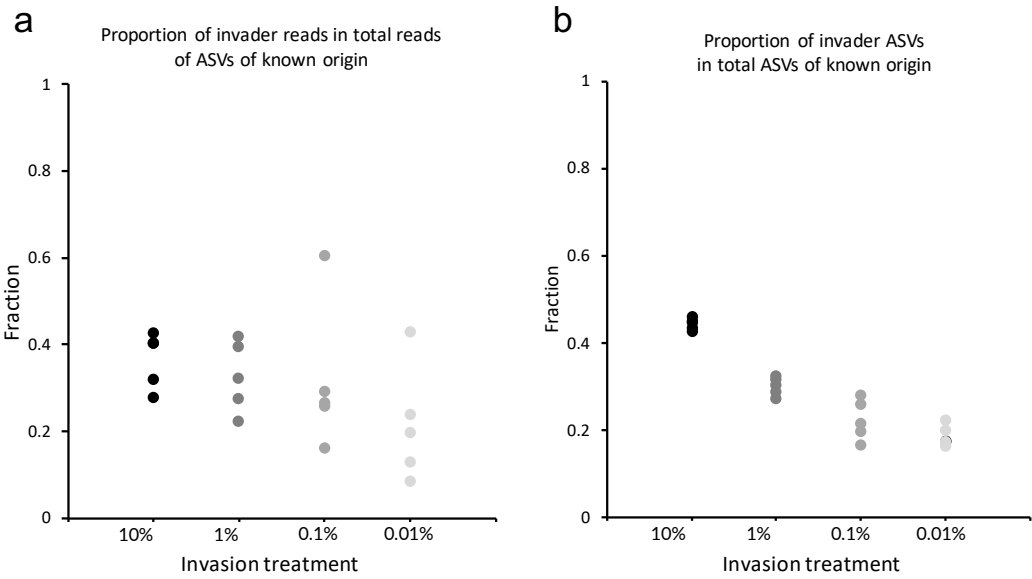
457   Figure 2: (A) Fraction of invader ASV reads in the total number of known-origin ASV reads

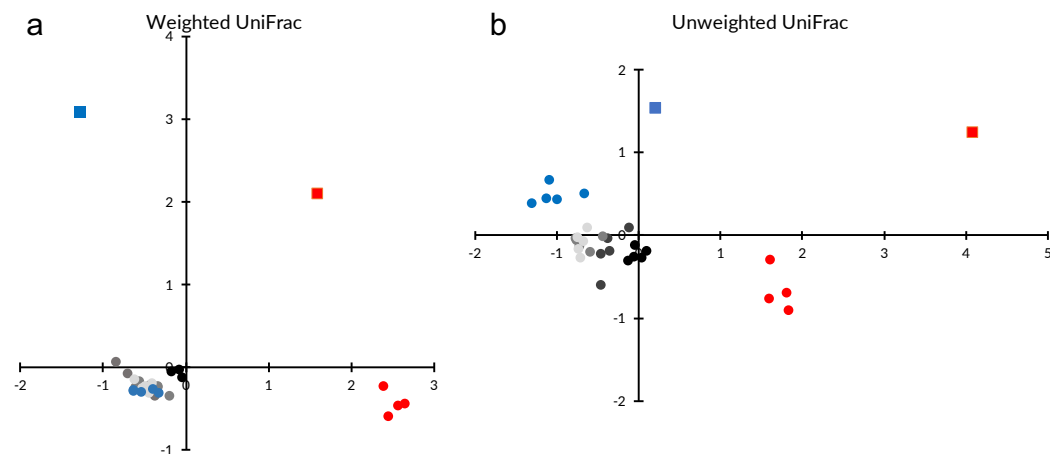458   (invader abundance); (B) Fraction invader ASVs in total known origin ASVs (invader diversity).

459

460   Figure 3: NMDS plot of: Weighted (A) and Unweighted (B) UniFrac of all the treatments. Red

461   square: High producer ancestor; Blue square: Low producer ancestor; Red circles: High

462   producer; Blue circles: Low producer, Black to light grey: Invasion treatments: in order from
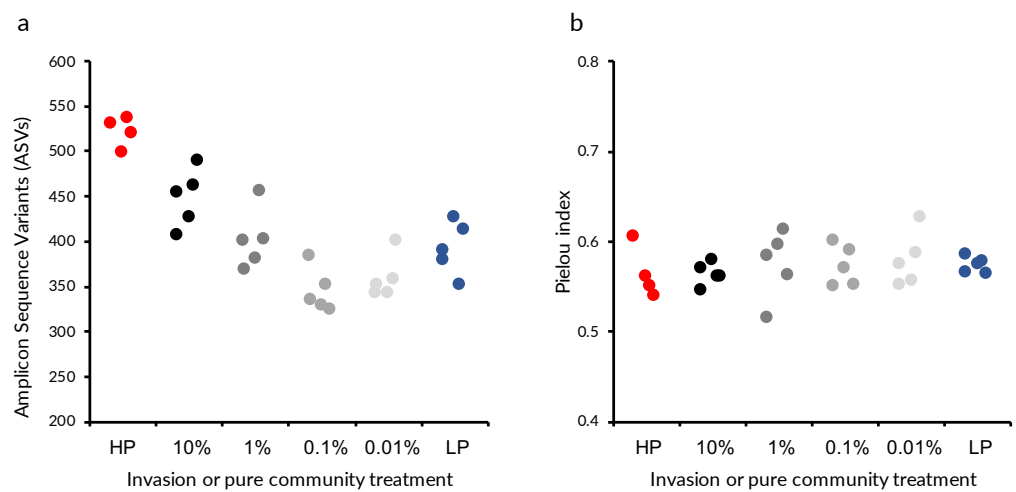
463   darkest to lightest: 10%, 1%, 0.1%, 0.01%

464

465

466

Figure 4: (A) Number of Amplicon Sequence Variants per sample (richness). (B) Pielou index (evenness).
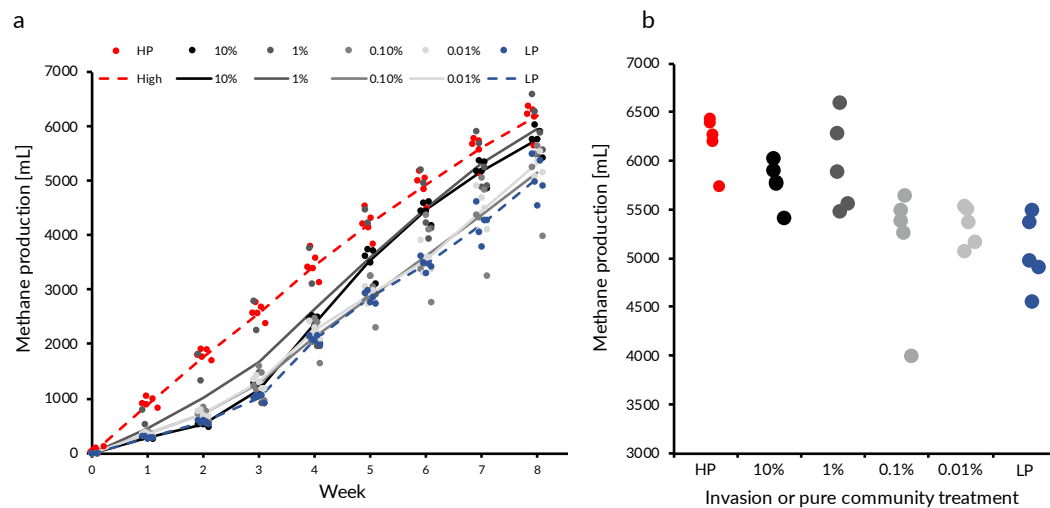
472

Figure 5: (A) Mean gas production over time. Points are individual replicates, while lines show treatment means. (B) Cumulative gas production at week 8.