SUPPLEMENTARY INFORMATION FOR:

Protection and enrichment: How different carbonaceous biofilm supports improve methane yield from encapsulated anaerobic microorganisms

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Recipe for synthetic wastewater

Table S 1: Recipe for the synthetic wastewater used in the study

Ingredients	Concentration (g/L)
Polysorbate (tween 80)	0.96
Gelatin	3.75
Starch	1.75
Casamino Extract	0.08
Yeast Extract	0.08
Ammonium Chloride	0.15
Sodium Bicarbonate	0.1
Sodium Phosphate	0.025
Potassium Phosphate	0.03
Magnesium Chloride	0.04
Calcium Chloride	0.06

Properties of Biochar

Table S 2: Properties of biochar used in the study

Property	Unit	Biochar	PAC
pH_{H2O}		9.18	6-8
C	%	73.2	
$C_{organic}$	%	73.1	
N	%	0.64	
$C_{organic}/N$	%	114	
H	%	1.03	
O	%	5.7	
H:C		0.17	
O:C		0.06	
Ash	%	19.4	≤ 1
EC	µS/cm	782	370000
SA	m2/g	231	1000
Ca	%	4.9	0.03
Fe	%	0.27	0.02
K	%	0.84	
Mg	%	0.33	0.02
B	mg/kg	36	
Cd	mg/kg	<0.2	
Cr	mg/kg	10	
Cu	mg/kg	16	≤ 15
Hg	mg/kg	<0.07	
Mn	mg/kg	310	
Na	mg/kg	830	
Ni	mg/kg	8	
P	mg/kg	1400	
Pb	mg/kg	<2	≤ 10
S	mg/kg	400	
Si	mg/kg	22000	
Zn	mg/kg	45	≤ 5
PAHs	mg/kg	6.7	
BET surface area	m^2/g	231	

C_{org} =organic carbon, EC = electrical conductivity,
SA = surface area, PAHs = polycyclic aromatic hydrocarbons (sum of the EPA's 16 priority pollutants)**(1)**

Gene extraction and qPCR method

Three beads from each treatment/reactor were sampled and stored at -20°C, and DNA was later extracted using the MP bio FastDNA™ SPIN Kit for Soil. Approximately 0.15g of each bead was combined with 978µL sodium phosphate buffer and 122uL MT buffer in lysing matrix and processed in an MP bio FastPrep-24™ 5G homogenizer, two times at 60m/s for 40s. The rest of the DNA extraction was performed according to the manufacturer's methods, and DNA was eluted into 50µL of DES solution. The DNA extraction process was performed on one sample of 400uL molecular biology grade water (Sigma, W4502) to serve as an extraction blank.

A SYBR green qPCR assay was used to quantify abundance of total bacteria (16s rRNA, V3 region) and methanogenic bacteria (methyl coenzyme A reductase, mcrA). Real-time qPCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) with a final reaction volume of 25 µl. Reactions for 16s quantification were comprised of 12.5µL SsoAdvanced SYBR Green Supermix, 0.6µL of 10µM 338-F primer, 0.6µL of 10µM 518-R primer, 0.5 µL of 20mg/L bovine serum albumin (Thermo Scientific™, B14), 9.8µL of molecular biology grade water (Sigma, W4502), and 1µL of sample. Reactions for mcrA quantification were comprised of 12.5µL SsoAdvanced SYBR Green Supermix, 2µL of 10µM mlas-F primer, 2µL of 10µM mcrA-R primer, 0.5 µL of 20mg/L bovine serum albumin (Thermo Scientific™, B14), 7µL of molecular biology grade water (SIGMA, W4502), and 1µL of sample. Thermal cycling was performed using a Bio-Rad CFX Connect Real-Time System according to the protocol: initial denaturation at 95°C for 3min followed by 45 cycles of: denaturation at 95°C for 15s and annealing/extension at 60°C for 45s. Melt curves were generated at the end of cycling using a temperature gradient from 60°C to 95°C at 5°C increments for 10s each.

Standard oligonucleotide sequences (gBlocks) for each primer set were purchased from IDT, hydrated with molecular biology grade water (Sigma, W4502), and serially diluted to concentrations ranging from 10^{10} copies/µL down to from 1 copy/µL. Standard curves of log gene copy number vs cycle number (C_q) were generated by qPCR of serially diluted gBlock samples performed in triplicate. The standard curves were used to calculate gene copy concentrations in each sample. The reported gene copies/uL for each sample are the average of three technical replicate qPCR reactions performed per bead sample. Results are presented as the ratio of mcrA to 16s gene copies detected for each sample. Reaction efficiencies were calculated from standard

curves according to Equations 1-3. Efficiencies of 16s and mcrA qPCR reactions used in this study were 0.932 - 0.947 and 0.935 - 0.976, respectively (**Table S3**).

Equations for analyzing qPCR standard curves, where $N_n =$ *the number of PCR amplicons after* n *cycles,* $N_0 =$ *is the starting number of gene copies in a reaction, and E is the PCR efficiency. Using* ***Eq 2****., n is the slope of the standards curve, which can then be used in* ***Eq 3****. to derive E*(2)

$$N_n = N_0 \times (1 + E)^n$$

Equation 1. PCR amplification

$$log(N_n) = log(N_n) + n(1 + E)$$

Equation 2. Linearized PCR amplification

$$E = 10^{-1/n} - 1$$

Equation 3. PCR efficiency

Table S 3: PCR amplification efficiencies (E), standard curve regression coefficients and information of primer sequences and gBlock standards for 16s rRNA and amoA genes used in qPCR assays.

Gene	Primer sequence (5' → 3')	Primer T_m	gBlock standard sequence	E	R^2	Linear range
16S rRNA *Bacteria* *V3 region*	338-F: 5'-TCCTACGGGAGGCAGCAG 518-R: 5'-ATTACCGCGGCTGCTGG (3,4)	65.1°C 64.6°C	CGGTCCAGAC**TCCTACGGGAGGCAGCAG**TGG GGAATATTGCACAATGGGCGCAAGCCTGATG CAGCCATGCCGCGTGTATGAAGAAGGCCTTCG GGTTGTAAAGTACTTTCAGCGGGGAGGAAGG GAGTAAAGTTAATACCTTTGCTCATTGACGTTA CCCGCAGAAGAAGCACCGGCTAACTCCGTG**CC AGCAGCCGCGGTAAT**ACGGAGGGTGCAAGCG TTA	0.932 - 0.947	0.999 – 1.00	10^3 - 10^9 copies per reaction
mcrA	mlas-F: 5'-GGTGGTGTMGGDTTCACMCARTA mcrA-R: 5'-CGTTCATBGCGTAGTTVGGRTAGT (5)	63.0-70.0°C, mean = 65.5°C 63.2-68.5°C, mean = 65.6°C	CTGTATGCTCTACGACCAGATCTGGCTCGGAT CCTACATGTCC**GGTGGTGTCGGATTCACACAG TAT**GCAACAGCTGCATACACCGATGATATCCT CGACAACAACACCTACTACGACGTTGACTACA TCAATGACAAGTACAACGGTGCTGCAACTGTC GGTAAGGACAACAAGGTAAAGGCAAGCCTCG AAGTCGTAAAGGACATAGCAACCGAATCCACA CTCTATGGTATCGAGACCTACGAGAAGTTCCC GACTGCCCTTGAAGACCACTTCGGTGGGTCCC AGAGAGCAACCGTGCTCGCAGCTGCAGCCGGT GTTGCATGCTCTCTTGCTACAGGAAACGCCAAT GCCGGTCTCTCAGGTTGGTACCTCTCCATGTAC CTGCACAAGGAAGCATGGGGCAGACTCGGATT CTTCGGTTTCGACCTGCAGGACCAGTGCGGTGC CACAAATGTTCTGTCCTACCAGGGCGACGAAGG TCTCCCAGACGAACTCCGTGGTCCAAA**CTACCC CAACTACGCAATGAACG**TTGGTCACCAG	0.935 - 0.976	0.998 - 0.999	10^2 – 10^{10} copies per reaction

Gas production by the capsules

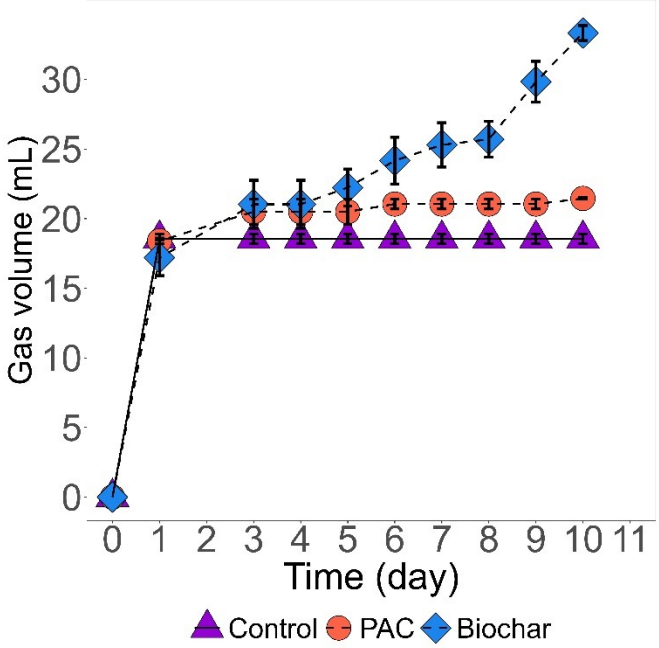

Figure S 1: Volume of gas produced by capsules containing freely suspended microorganisms (control), biofilms grown on 2% w/v PAC, and the biofilms grown on 2% w/v biochar. Symbols show the average of triplicate samples, and the error bars are standard deviations. Lines are to guide the eye.

Loss of capsule stability due to addition of PAC

(a) (b)

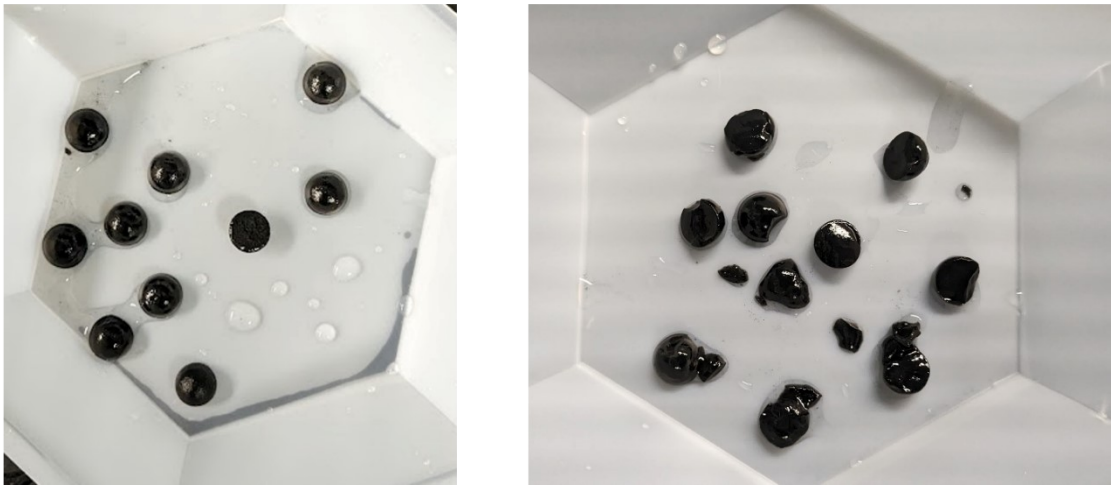

Figure S 2: (a) Biochar-amended PEG capsules after 10 days of incubation (b) PAC-amended PEG capsules after 10 days of incubation.

Methane production activity of the capsules

Table S 4: Methane production per mcrA gene copies and 16S rRNA gene copies values for the different capsule types

Capsule type	$\frac{fmol\ CH_4}{mcrA\ gene\ copies}$	$\frac{fmol\ CH_4}{16S\ rRNA\ gene\ copies}$
Control	782.6 ± 45.2	257.5 ± 36.4
PAC	895.4 ± 132.1	394.3 ± 5.9
Biochar	3081.3 ± 517.98	3056.2 ± 1252.3

References

1. Harter J, Guzman-Bustamante I, Kuehfuss S, Ruser R, Well R, Spott O, et al. Gas entrapment and microbial N2O reduction reduce N2O emissions from a biochar-amended sandy clay loam soil. Sci Rep. 2016 Dec;6(1):39574.

2. Kralik P, Ricchi M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. Front Microbiol [Internet]. 2017 Feb 2 [cited 2023 Apr 12];8. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00108/full

3. Johnston J, LaPara T, Behrens S. Composition and Dynamics of the Activated Sludge Microbiome during Seasonal Nitrification Failure. Sci Rep. 2019 Dec;9(1):4565.

4. Nübel U, Garcia-Pichel F, Muyzer G. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol. 1997 Aug;63(8):3327–32.

5. Steinberg LM, Regan JM. *mcrA* -Targeted Real-Time Quantitative PCR Method To Examine Methanogen Communities. Appl Environ Microbiol. 2009 Jul;75(13):4435–42.