Electronic Supplementary Material (ESI) for Environmental Science: Nano.

A high-throughput method to characterize the gut bacteria growth upon engineered nanomaterial treatment

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Supplementary Information

Structure of method section:

2. Method
2.1 Bacterial strains, *in vitro* gut microbiome, and cultivation conditions
2.2 Nanomaterials — Rational for choice of nanomaterial and their characterisation.
2.3 Development of high-throughput DNA based quantification (DBQ) assay
Step 1: Plate layout and design of controls.
Step 2: Sample treatment.
Step 3: Partial DNA extraction.
Step 4: PicoGreen assay.
— Detailed description of the method protocol
2.4 Method optimization
Testing on pure DNA adsorption by ENM.
Testing on effect of lysis buffer.
Testing on biomass change after washing.
Testing on plate layout effect and DNA extraction process optimization.
— Evaluation of factors that may affect quantification results.
2.5 Standard curve construction and limit of detection calculation — Method validation by standard curve construction.
2.6 Dose response for the growth of *E. coli* with chitosan — Example of application.

Bullet form of method protocol

(a) Sample preparation
1. Pre-sonicate 10x ENM stock solution
2. Pre-spike 25* µl 10x ENM stock to wells for test assay and blank controls in deep 96 well plate with triplicates
3. Add 25* µl water to wells for negative controls in deep 96 well plate with triplicates
4. Prepare bacterial culture at designed concentration for toxicity assay or standard curve construction
5. Add 225* µl bacterial culture to wells for test assays and negative controls and mix
6. Add blank medium/buffer to wells for blank controls and mix
7. Incubate samples when needed

(b) Sample Treatment
8. Post-spike ENM stock solutions to negative controls for toxicity assay
9. Centrifuge at 4000 rpm (max speed) for 5 min and remove supernatant by flipping over plate
10. Add 250 µl 0.85% NaCl and mix
11. Repeat step 9 and 10
12. Centrifuge at max speed and remove supernatant by flipping over plate
13. Cover the plate and store the pellet samples in -20 °C temporarily if more than one plate is expected in a batch

(c) Partial DNA extraction

13. Add 250 µl lysis buffer per well to pellet samples, completely mix the samples with lysis buffer and cover the plate with caps
14. Freeze samples in liquid nitrogen for 1 min
15. Thaw the samples in boiling water for 1 min
16. Repeat step 14 and 15
17. Add 250 µl TE buffer and mix
18. Cover the plate with caps and sonicate for 15 min
19. Centrifuge at max speed for 5 min
20. Pipette transfer 150 µl supernatant to a clean transparent 96-well plate

(d) PicoGreen assay

21. Dilute the supernatant sample 50-200 times in TE depending on sample concentration (trial experiments are needed)
22. Transfer 50 µl diluted sample to black 96-well plate with duplicates
23. Prepare λDNA standard from 0 to 500 ng/ml
24. Transfer 50 µl of each λDNA standard to black 96-well plate with duplicates
25. Prepare PicoGreen working reagent (200x dilution of dye)
26. Add 50 µl PicoGreen working reagent per well to the diluted samples and λDNA standards in black 96-well plate
27. Incubate the black 96-well plate at dark for 8-10 min
28. Take fluorescence readings of the black 96-well plate(s) at 485/535 nm with microplate reader
29. Plot fluorescence vs. DNA standard curve and calculate the concentration of diluted samples
30. Multiply the concentration with dilution factor (50-200) to get the final DNA concentrations.

*: volume of ENM and bacterial culture may be increased when culture density is too low to get accurate results.

Results on graphene oxide (GO) characterization:

Based on atomic force microscopy (AFM) measurements, GO was organized in single layers with a lateral size of 242 ± 118 nm and a thickness of 0.8 ± 0.1 nm. As measured by XPS, the C/O ratios of GO was 1.53. The endotoxin level is less than 0.5 EU /mg and the microbiological result is 0 CFU/g , demonstrating the sterility of the material. Complete characterization data, including atomic force microscopy (AFM), XPS, endotoxin and microbiological test are presented in Supplementary Table S1.

Table S1: Physiochemical characterization and sterility assessment of pristine GO

Atomic force microscopy (AFM)

Summary of statistical data on lateral size (L) and thickness distributions of GO as measured by AFM.

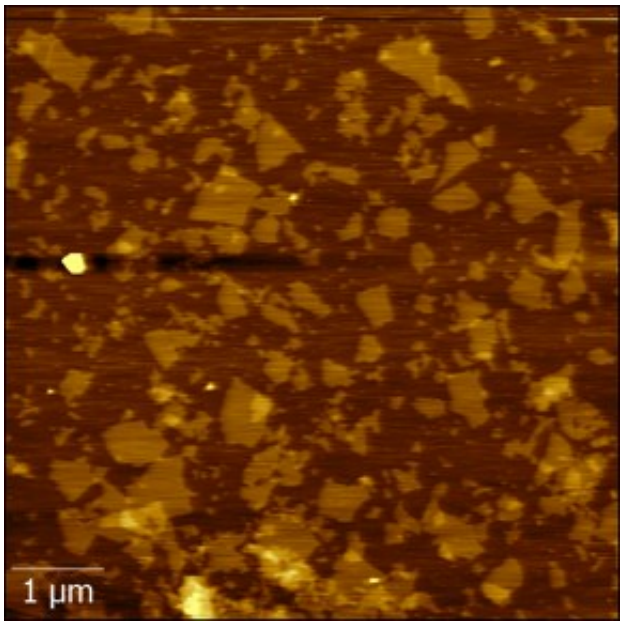
N*	mean ± SD (nm)	min. (nm)	25th % (nm)	median (nm)	75th % (nm)	max. (nm)	geom. mean (nm)	σ_g **
166	**242 ± 118**	26	158	212	305	696	214	1.68

*N: number of particles; **σ_g: geometric SD factor

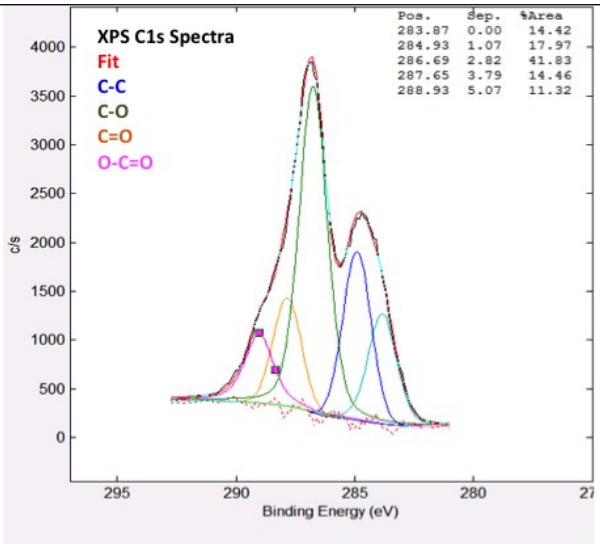
Statistical data on thickness (h) distributions of GO as measured by AFM.

N*	mean ± SD (nm)	min. (nm)	25th % (nm)	median (nm)	75th % (nm)	max. (nm)	geom. mean (nm)	σ_g **
39	**0.8 ± 0.1**	0.6	0.7	0.8	0.8	1.0	0.8	1.1

* N: number of particles; ** σg: geometric SD factor



Single layer thickness 0.8 - 1.2 nm
Avergae lateral size (N=166): 242 ± 118

<table>
<tr><td>X-Ray Photoelectron Spectroscopy (XPS)</td><td> 

<table>
<tr><td>Element</td><td>Peak</td><td>Atomic percentage (%)</td></tr>
<tr><td>Carbon</td><td>C1s</td><td>62.26</td></tr>
<tr><td>Oxygen</td><td>O1s</td><td>37.17</td></tr>
<tr><td>Sulfur</td><td>S1s</td><td>0.57</td></tr>
</table></td></tr>
<tr><td>Endotoxin (EU mg^{-1})</td><td>< LOD
Endotoxin assay LOD: 0.5 EU mg^{-1}</td></tr>
<tr><td>Microbiological test (CFU g^{-1})</td><td>0</td></tr>
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In-vitro gut reactor inoculation process

Before inoculation, the whole reactor chamber with medium was autoclaved for sterilization. Then the outlet for gas collection was open and the medium inside was flushed with N_2 through the N_2 purge inlet connected with a 0.22 µm gas filter for at least an hour. The fresh fecal sample from one healthy donor was transferred to anaerobic chamber within 1 hr. Approximately 20 g sample was mixed with growth media and settled for 5 min to remove large particles. Then the supernatant was transferred to the pre-reduced reactor medium for inoculation. The N_2 purging continued for several hours after inoculation. Then the N_2 purge inlet was closed, and the gas collection outlet was connected with an empty gas bag to collect any gas production. To maintain the anaerobic condition of the influent, the medium bottle was immediately connected with a N_2 gas bag while the medium inside was still hot after being autoclaved. Effluent bottle was also autoclaved and flushed with N_2 through a filtered inlet. After N_2 flushing, another empty gas bag was then connected to avoid oxygen leakage to the system.

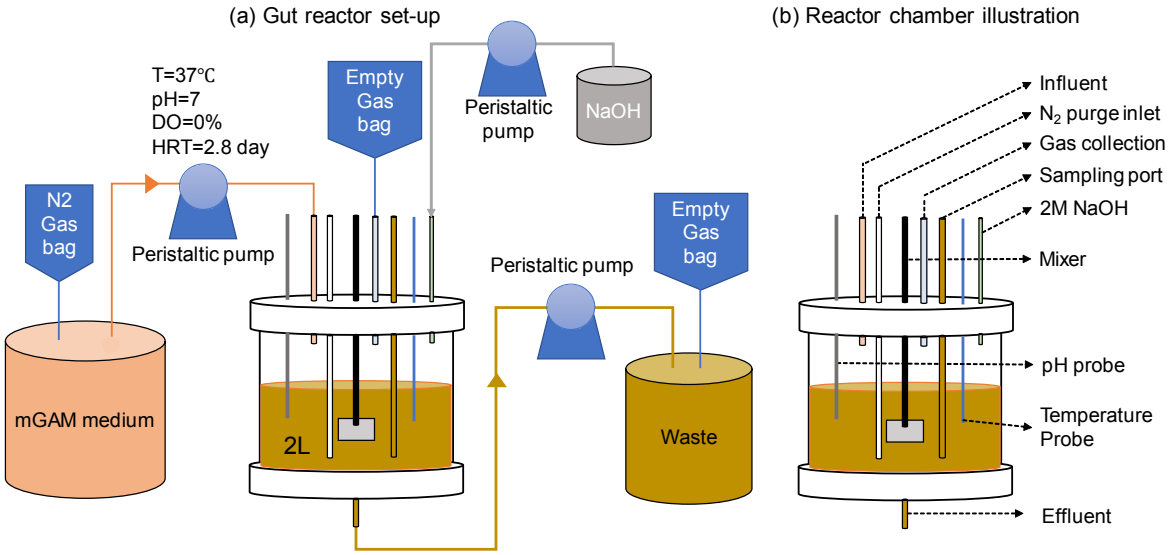

Figure S1 Schematic illustration of (a) reactor set-up and (b) reactor chamber layout which illustrated all the inlets, outlets, and probes.

Background noise from medium component

The rich medium contained a lot of organics and proteins which would interfere with PicoGreen staining and most of the components could be washed away with the washing step. However, with the addition of certain ENMs, some medium components may bind with the ENM and could not be removed by the washing step (comparing between a and b, d, and e). Among the tested ENMs, chitosan had the strongest background effect (data not shown), and therefore was used to elaborate the background noise level. Since the ENM concentration and medium components are the same for all testing samples in quantification experiment, we could accurately determine the genomic DNA concentration by subtracting the value of the blank control (group b) from the measured result (group e).

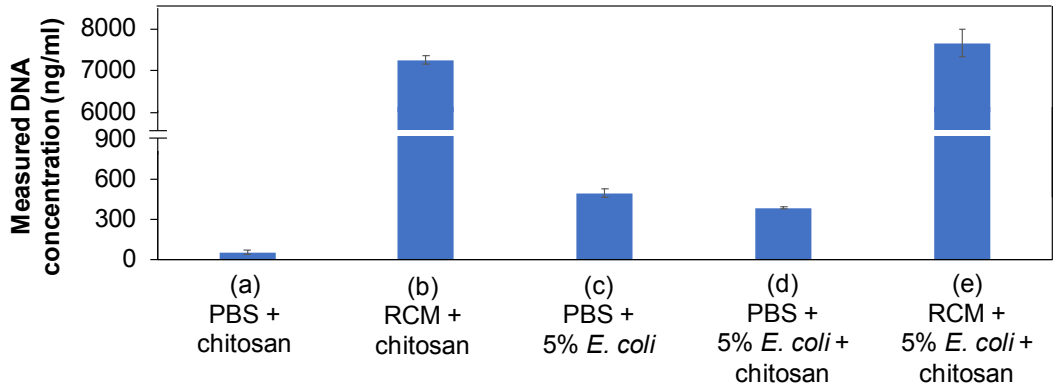

Figure S2 Comparison of background noise of chitosan ENM with different substrate. Five different substrate + ENM + bacteria combinations were tested: (a) PBS with chitosan and no bacteria; (b) RCM with chitosan and no bacteria; (c) PBS with 5% *E. coli* and no ENM; (d) PBS with 5% *E. coli* and chitosan ENM; and (e) RCM with 5% *E. coli* and chitosan ENM. The data presented are the average and standard deviation of triplicates (n=3).

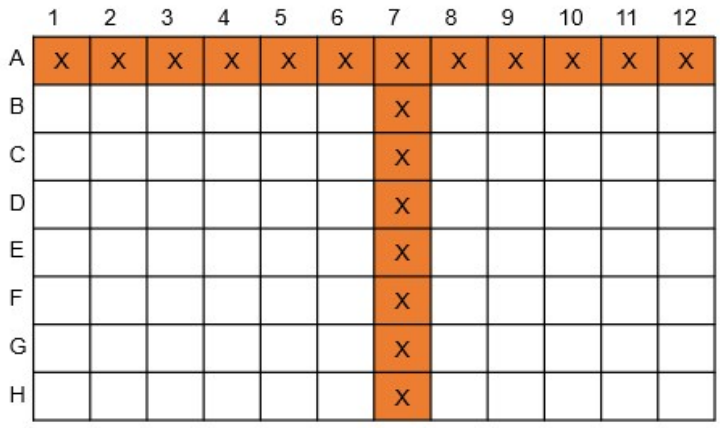

Figure S3 Design of 96-well plate layout for the position effect experiment. Sample replicates were added to Row A and Column 7 of the 96-well plate which represented the edge and center area of a plate.

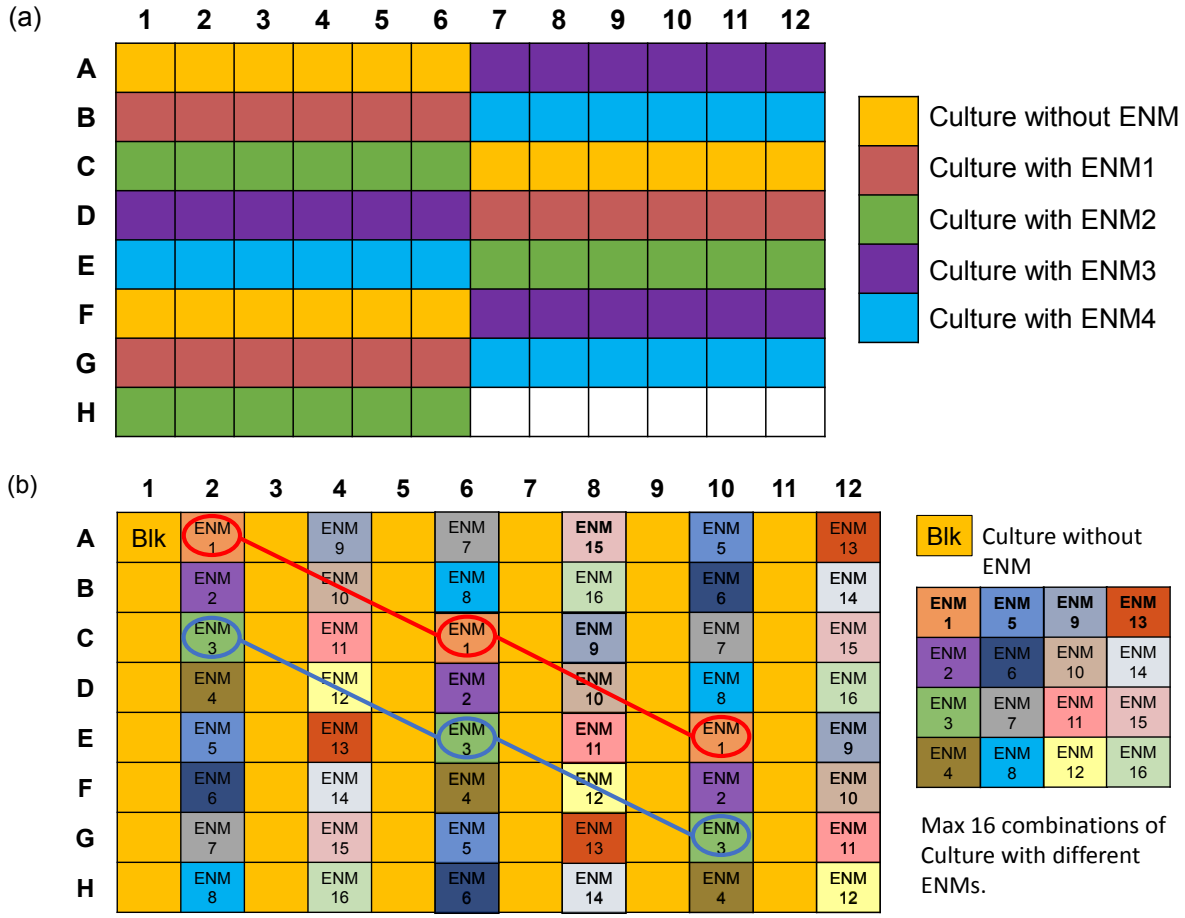

Figure S4 Design of 96-well plate layout for (a) standard curve construction and (b) quantification of max 16 “culture + ENM” combinations. The wells for culture without ENMs in layout (b) are to be post-spiked with respective ENMs immediately after incubation. The layout design was aimed to achieve even distribution of replicates.

Characterization of ENM aggregation

ENMs (500 µg/mL) dispersed in DI water by employing sonication energy of 354 J/mL with probe sonicator (QSonica, US). Thereafter, the ENMs was added to the different medium to the final concentration of 100 µg/mL. The ENMs' hydrodynamic diameter (DH) and polydispersity index (PDI) in the various mediums were analyzed with Dynamic Light Scattering (DLS, Malvern UK) at 0h and 24h post sonication. Aggregation of the ENMs over the course of 24h was determined through the change in the hydrodynamic diameter (ΔDH), in which ≤30% change in the DH was set as the threshold to indicate the formation of stable aggregates. Measurement was done in triplicate and the data presented are mean ± standard deviation (S.D.).

ENMs were detected to form moderately homogenous (PDI = 0.14 – 0.26) suspensions in DI water with registered hydrodynamic size ranging between 177 to 2738 nm. In general, significant hydrodynamic size increase was detected when the ENMs were dispersed in buffers (i.e. saline, PBS) and culture mediums (i.e., RCM, mGAM), suggesting the aggregation of the materials in the relevant mediums that were used in the studies.

In general, SiO_2 and GO were found to produce a more stable aggregates, as evidenced from minimum to moderate change in the hydrodynamic size over the course of 24 hr. Whereas, both chitosan and CNF were noted to be prone to aggregate over the course of time.

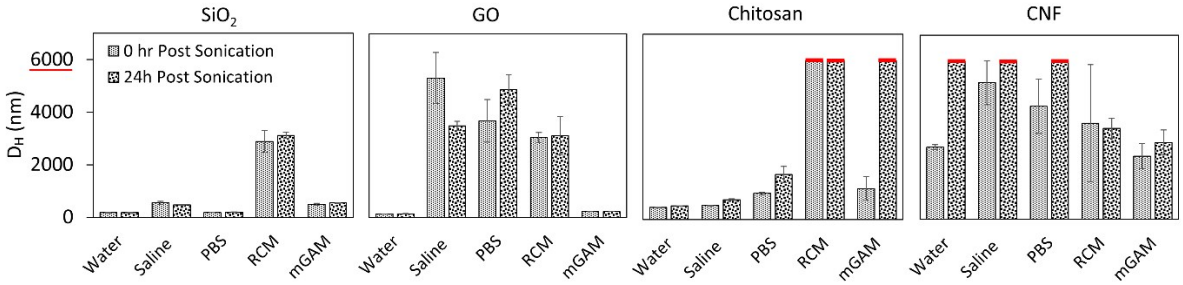

Figure S5 Hydrodynamic diameter (D_H) of the ENMs in relevant dispersants utilized in the study. Instrument detection limit: 6000 nm. Error bars are standard deviation of triplicates.

Standard curves of pure strains

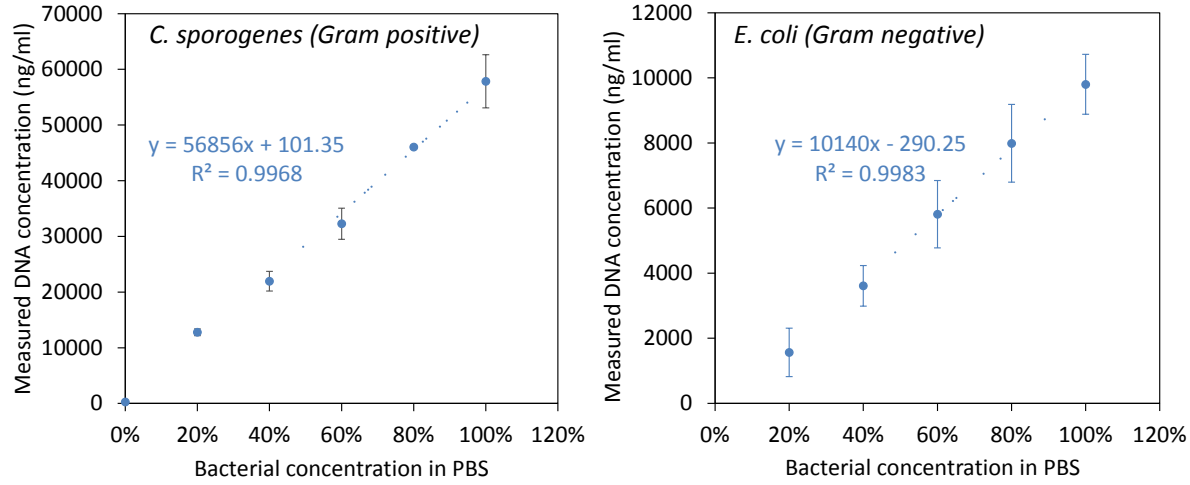

Figure S6 Standard curves of *C. sporogenes* (gram positive) and *E. coli* (gram negative). R^2 is the correlation coefficient. Error bars represent the standard deviation of triplicates.