Supplementary Information (SI) for Analyst.
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Electronic supplementary material (ESI)

CO_2-based micro-respirometry for measuring bacterial load under aerobic and anaerobic conditions

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S1. Growth and plate counting medium

Preparation of the liquid and solid growth media for use with E. coli.

The liquid growth medium used in this work was a nutrient-rich broth (NB), prepared by adding 5 g casein yeast peptone, 10 g glucose and 5 g sodium chloride to a 1 L borosilicate glass reagent bottle containing 1 L water. Once all the ingredients were fully dissolved, the growth medium was sterilized by autoclaving at 121 °C for 15 minutes. The solid agar growth medium used for the plate count method (PCM) was prepared by adding 10 g agar to 1 L of the liquid growth medium, autoclaved at 121 °C for 15 min, and then allowed to cool to 52 °C in air. Then, the hot liquid agar was poured into the large Petri dishes used for the aerobic PCM.[1] Subsequently, the plates were allowed to cool to room temperature, and then refrigerated until needed.

References

1. ISO 4833–2, ISO 4833–2:2013, Microbiology of the food chain- horizontal method for the enumeration of microorganisms- part 2: colony count at 30 °C by the surface plating technique, International Organization for Standardization, Geneva, 2013.

S2 Preparation of stock dispersion of *E. coli*

Preparation of E. coli stock cultures

To produce a primary stock culture, a KWIK-STIK containing a pure population of *E. coli* was gently swabbed across the surface of the solid agar in a 90 x 15 mm Petri dish.[1] The inoculated culture plate was immediately incubated at 30 °C overnight, after which there was visible growth of single colonies of bacteria; subsequently, the plate was wrapped in Parafilm® and refrigerated until needed.

The process of making an overnight stock of a liquid culture of *E. coli* was as follows: a single colony of the bacterium was taken from the primary culture plate using a sterile inoculating loop and suspended in 10 mL of NB in a 15 mL Falcon® tube, which was then incubated overnight at 30 °C. The loading of the resulting overnight culture, *ca.* 10^8 CFU/mL, was confirmed using PCM.[2] Serial 1-in-10 dilutions of the overnight bacterial culture in NB were used to produce suspensions of initial inoculum concentrations of *ca.* 10^8-10^1 CFU/mL of the bacteria; these dilutions were prepared fresh on the day of use, and 1 mL samples of each inoculum in 9 mL NB were used in creating a calibration curve for the %CO_2-μR system. In generating the calibration curve for *E. coli* under *anaerobic* conditions, 50 mg sodium sulfite were added to the Falcon® tube containing 1 mL inoculum/ 9 mL NB before incubation.

Aerobic PCM was carried out by inoculating 3 agar plates with 0.1 mL of the assumed 'ca. 10^3 CFU/mL' dilution, which was spread across the plate with an L-spreader; the plates were incubated at 30 °C overnight. After this period, there was visible growth of single colonies of bacteria on each plate, which could then be counted, an average of the 3 plate counts taken, and the results used to estimate the microbial load of the original overnight culture stock.[2]

References

1. Microbiologics KWIK-STIK™, https://www.microbiologics.com/item-type/Product/product-format/KWIK-STIK-2-Pack,KWIK-STIK-6-Pack, (accessed July 2024).

2. ISO 4833–2, ISO 4833–2:2013, Microbiology of the food chain- horizontal method for the enumeration of microorganisms- part 2: colony count at 30 °C by the surface plating technique, International Organization for Standardization, Geneva, 2013

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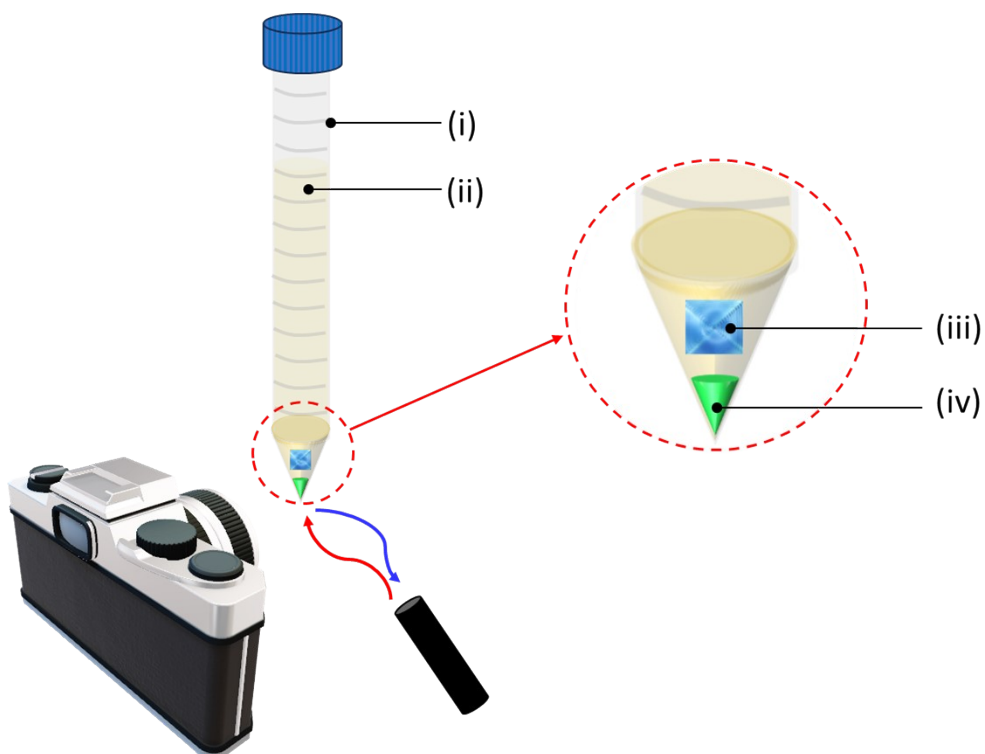
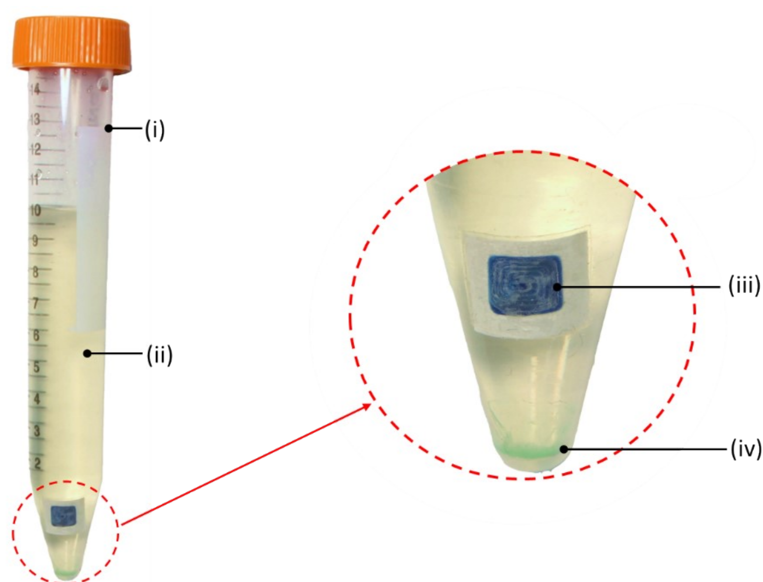
S3. Falcon™ tube 'bioreactor' with O_2 and CO_2 sensors

A schematic illustration of the typical %CO_2-µR 'bioreactor' used in this work is given in Fig. S1 and comprised a 15 mL Falcon® tube with a luminescence-based O_2 indicator (Oculer Ltd., Tipperary, Ireland) set in its base. To this was added a 3D printed XB/LDPE %CO_2 indicator, printed on Tyvek™ and secured in place using surgical tape. Typically, this Falcon® tube 'bioreactor' was filled with 9 mL of the relevant growth medium and 1 ml of the inoculum under test, shaken and then incubated (in a Heratherm™ Compact Microbiological Incubator (Thermo Fisher Scientific, Massachusetts, USA) at 30 ^{o}C over time, *t*, during which the apparent absorbance, *A'*, of the XB/LDPE %CO_2 indicator, and the lifetime of the luminescent O_2 indicator were monitored simultaneously.



(a)



(b)

Fig S1. (a) Schematic and photographic images of the typical 'bioreactor' used in this work, comprising (i) 15 mL a Falcon™ tube, (ii) growth medium (9 mL) plus inoculum (1 mL), (iii) a 3D-printed, colourimetric XB/LDPE CO_2 indicator, and (iv) luminescence based O_2 indicator beads. The apparent absorbance, *A'*, of the CO_2 indicator, and the lifetime, τ, of the O_2 indicator were monitored simultaneously using digital photography and a fibre-optic O_2 monitor, represented by the camera and measuring probe showing the excitation (red) and detected luminescence emission (blue), as illustrated in (a).

S4 Response and recovery time of the XB/LDPE indicator

A 3D printed xylenol blue (XB) CO_2 indicator, printed on Tyvek®, was sellotaped on the inside of a 1 cm cuvette, filled with nutrient broth. The cuvette was placed inside an incubator (Heratherm™ Compact Microbiological Incubator) set to 30 °C and alternating gas streams of 5 % CO_2 and air and were sparged through the cuvette. The colour change of the indicator was monitored photographically, and from the photographic images of the indicator, values of the apparent absorbance, *A′*, were calculated as a function of time. The results of this work are illustrated in Fig. S2.

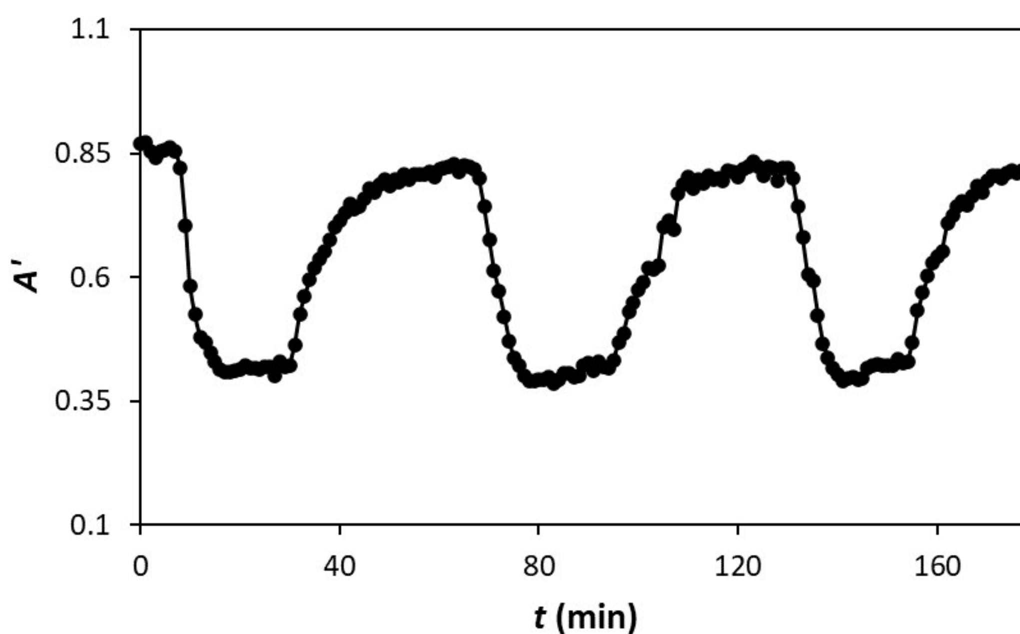


Fig. S2. Response and recovery spectra of the 3D printed XB CO_2 indicator in nutrient broth, sparged with an alternating stream of 5% CO_2 and air at 30°C revealing a 90% response time = 8.0 min and 90% recovery time = 14 min.

S5. Reproducibility of the 3D printed CO_2 indicator

The reusable nature of the 3D printed XB CO_2 indicator used in %CO_2-µR was demonstrated by recording the *A′* vs t profiles for 10 otherwise identical %CO_2-µR runs using 10 different 3D printed XB/LDPE CO_2 indicators in NB inoculated with 10^4 CFU/mL of *E. coli*. The results of this work are illustrated in Fig. S2 and reveal an average TT value of 7.43 ± 0.18 h.

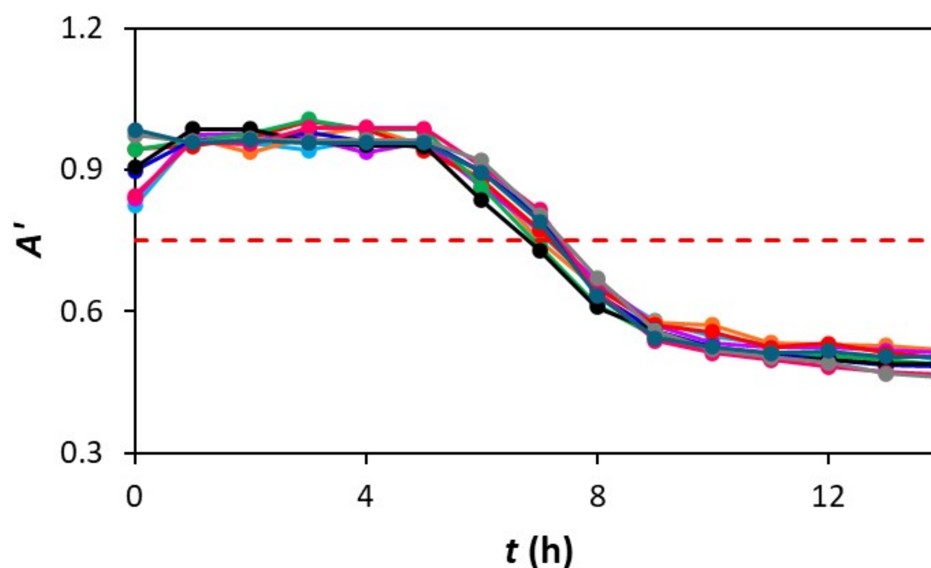


Fig. S3. Apparent absorbance, *A′*, vs incubation time, *t*, profiles for 10 different 3D printed XB CO_2 indicators exposed to a 10^4 CFU/mL culture of *E. coli*, recorded at 30 °C. This data reveals an average TT value of 7.43 ± 0.18 h.

S6. %CO_2-μR results, using *E. coli*, under aerobic and anaerobic conditions

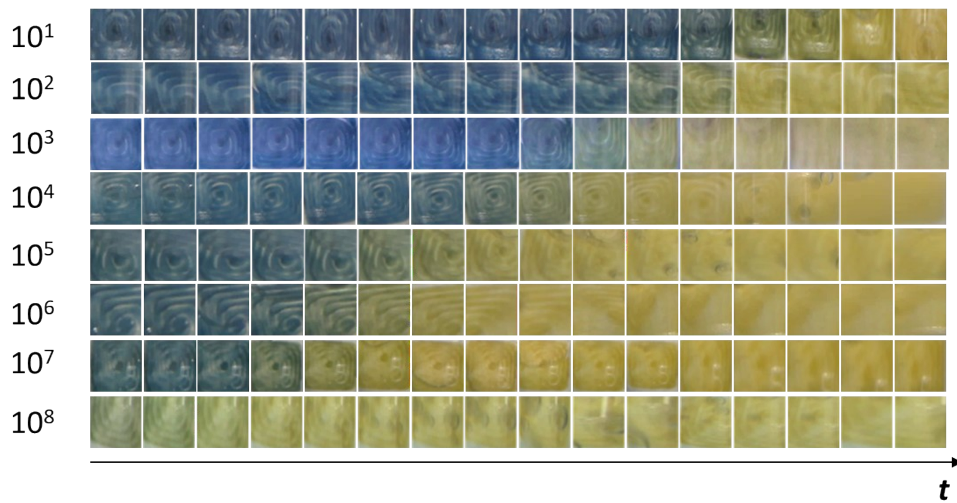


Fig. S4. Photographs of the XB/LDPE %CO_2 indicator in inoculated NB recorded as a function of incubation time, *t*, at 30 °C, every hour for 15 h, with initial inoculums (1 mL in 9 mL of NB) of 10^1 –10^8 CFU/mL of *E. coli*, respectively, recorded under *aerobic* conditions.

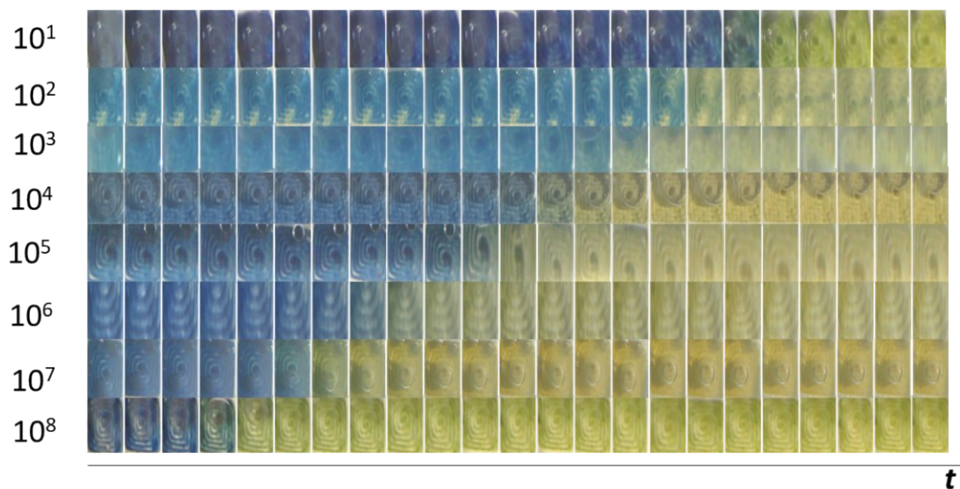


Fig. S5. Photographs of the XB/LDPE %CO_2 indicator in inoculated growth medium recorded as a function of incubation time, *t*, at 30 °C, every hour for 22 h, with initial inoculums (1 mL in 9 mL of NB) of 10^1 –10^8 CFU/mL of *E. coli*, respectively, recorded under *anaerobic* conditions.