Chapter 18

MiniBioReactor Arrays (MBRAs) as a Tool for Studying *C. difficile* Physiology in the Presence of a Complex Community

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Abstract

The commensal microbiome plays an important role in the dynamics of *Clostridium difficile* infection. In this chapter, we describe minibioreactor arrays (MBRAs), an in vitro cultivation system that we developed that allows for *C. difficile* physiology to be assayed in the presence of complex fecal microbial communities. The small size of the bioreactors within the MBRAs allows for dozens of reactors to be run simultaneously and therefore several different variables can be tested with limited time and cost. When coupled with experiments in animal models of *C. difficile* infection, MBRAs can provide important insights into *C. difficile* physiology and pathogenesis.

Key words *Clostridium difficile*, Microbiome, Bioreactors, In vitro, Microbial ecology

1 Introduction

Interactions with the commensal microbiome play an important role in the dynamics of *C. difficile* infection. To better interrogate these interactions in a controlled laboratory setting, in vitro cultivation models have been developed to assess *C. difficile* physiology in the presence of the microbiota. Freter and Wilson [1] used single-chamber continuous-flow bioreactors to cultivate hamster cecal communities capable of suppressing *C. difficile* growth in vitro and in *C. difficile* mono-associated mice. Freeman, O'Neill, and Wilcox adapted the three-stage continuous flow cultivation system described by MacFarlane, MacFarlane, and Gibson [2] to study *C. difficile* invasion of human fecal communities [3]. This model has been used extensively to study the impact of (1) different antibiotic regimens on *C. difficile* persistence (e.g., [4–6]), (2) co-infection with different *C. difficile* strains [7, 8], and [3] recurrence of *C. difficile* growth following vancomycin treatment [8].

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A modified version of this model, incorporating glass rods as a surface for biofilm formation, was also described [9]; this model provides a platform to study physiological differences between planktonic and biofilm-associated populations of *C. difficile*.

Building upon these studies, we developed a simplified, single chamber continuous-flow platform (mini-bioreactor arrays, MBRAs [10]) that allows for cultivation of many different fecal microbial communities in parallel. MBRAs use small volume reactor vessels (25 mL total volume, 15 mL operating volume) fabricated in blocks of six, paired with four 24-channel peristaltic pumps and two 60-spot stir plates to operate 48 reactors in parallel in a single anaerobic chamber. In our initial studies, we seeded MBRA with fecal samples pooled from 12 healthy donors, disrupted a subset of communities with clindamycin-treatment, and challenged treated and untreated communities with *C. difficile*. We found that untreated communities resisted invasion by *C. difficile*, whereas clindamycin-treated communities were susceptible. We used this reactor model to evaluate competition between *C. difficile* clinical isolates of different ribotypes and found that four ribotype 027 clinical isolates exhibited a competitive advantage over clinical isolates of four other ribotypes (ribotypes 001, 002, 014, and 053). These results were validated in a humanized microbiota mouse model of *C. difficile* infection.

Following these initial studies, we have tested the impact of several variations of our previously published MBRA protocol, including changes in fecal donors, composition of medium, retention time, clindamycin-dosage regimen, and *C. difficile* growth status (i.e., challenge with *C. difficile* spores or vegetative cells). In most cases, we have observed that our MBRA model was not impacted by these changes and note these alternative, unpublished protocol modifications throughout the chapter. Because a laboratory setup for the analysis of 48 MBRA communities requires significant capitol investment, we also describe a simplified bioreactor design with less need of specialized equipment.

2 Materials

2.1 Fecal Sample Processing for –80 °C Storage

1. Autoclavable laboratory spatulas.
2. Sterile 1.8–2.0 mL cryogenic tubes.
3. Phosphate buffered saline (PBS): 1 L contains 8 g sodium chloride, 0.2 g potassium chloride, 1.44 g sodium phosphate dibasic, and 0.24 g potassium phosphate monobasic. Adjust pH to 7.4 and filter-sterilize.
4. Portable laboratory scale with sensitivity to 0.1 g.
5. Incubator with capability for 65 °C incubation for 30 min.

6. TCCFA Agar (described in ref. [11]): For 1 L combine, 40 g proteose peptone #3, 5.75 g sodium phosphate monobasic, 2 g potassium phosphate dibasic, 2 g sodium chloride, 200 mg magnesium sulfate heptahydrate, 6 g D-fructose, and 15 g Bacto agar. Autoclave for 20 min, then add a filter-sterilized mix containing 1 g taurocholic acid sodium salt (Sigma), 250 mg D-cycloserine, and 16 mg cefoxitin.
7. Sterile laboratory spreaders, pipetman, and barrier tips.
8. Liquid nitrogen.
9. Low temperature laboratory freezer (−80 °C).

2.2 MBRA Assembly and Operation

1. MBRA are made from DMS Somos Watershed plastic by stereolithography with the previously described dimensions [10]. Our MBRA are manufactured by Proto Labs (www.protolabs.com, formerly FineLine Prototyping), but could be manufactured by other companies with similar capabilities. The CAD file for stereolithography is available upon request. Stereolithography does not produce threads for insertion of fittings within the MBRA; threads can be introduced with a ¼-28 hand tap tool (available from most hardware stores).
2. A 60-position magnetic stir plate is required, such as the MIXDrive60 produced by 2mag, which has inter-magnet spacing that matches the inter-reactor dimensions of the MBRA. One stir plate is needed for every four MBRA strips (24 reactors).
3. Two 24-channel peristaltic pumps with low flow-rate capabilities, such as the series 205S peristaltic pump with 24-channel drive produced by Watson-Marlow, are needed for each set of 24 reactors.
4. A heated anaerobic chamber with a hydrogen sulfide mitigation system and sufficient dimensions to contain the MBRA setup ($90 \times 80 \times 80$ cm ($l \times d \times h$)) is needed. The chamber must also provide an electrical power supply for peristaltic pumps and stir plate. If available, at least one pass-through port with butyl rubber stopper will allow removal of waste from the chamber. The atmosphere in the chamber should contain ≥5 % H_2, 5 % CO_2, and ≤90 % N_2 (5 % CO_2 is needed for adequate buffering of the bioreactor medium, which contains bicarbonate).
5. Tubing, fittings, and source bottle caps are available from Cole-Parmer and Kinesis Inc. and are described in Table 1. This table lists the tubing, fittings, and caps sufficient for a single run of 24 reactors. Although tubing is not reusable, fittings and bottle caps can be reused until noticeable signs of wear are detected.
6. One 500 mL, twenty-four 1 L, and four 2 L glass laboratory bottles with GL45 threaded caps.

Table 1
Tubing, fittings, and caps needed for assembly of 24 reactors

Tubing and fittings from Cole Parmer		
Part number	**Description**	**# packs**
EW-45505-82	Adapter, nylon, male luer to 1/4-28 thread, 25/pack	4
EW-45502-56	Female luer tee, Nylon, 25/pack	2
EW-45502-04	Female luer × 1/8″ hose barb adapter, Nylon 25/pack	3
EW-45502-02	Female luer × 3/32″ hose barb adapter, Nylon, 25/pack	1
EW-45505-04	Male luer with lock ring × 1/8″ hose barb adapter, Nylon, 25/pack	8
EW-45502-00	Female luer × 1/16″ hose barb adapter, Nylon, 25/pack	4
EW-96460-26	2-stop Tygon E-Lab, Tubing, 0.89 mm, 12/pack	2
EW-96460-30	2-stop Tygon E-Lab, Tubing, 1.14 mm, 12/pack	2
EW-06424-67	C-Flex Tubing, 1/8″ ID × 1/4″ OD, 25 ft/pack	3
Bottle caps, fittings, and solvent line from Kinesis		
00945Q-2	Omnifit Q-series two hole bottle cap	26
008NB32-KD5L	1/4-28 mm thread to barbed male adaptor (3.2 mm), 5/pack	5
008 T32-150-10	Tubing, PTFE, 1/8″ (3.2 mm) OD × 1.5 mm ID, 10 M	1

7. Holders for MBRA strips can be 3-D printed from ABS or any other suitable plastic. A CAD file for 3D printing that fits the current MBRA design and the 60-spot magnetic stir plate is available upon request.
8. 8 × 3 mm magnetic stir bars (1 per reactor) and 7 mm OD glass tubing precision seal rubber septa (1 per reactor).
9. Sterile 0.22 μm syringe filters.
10. Loctite instant mix epoxy (or equivalent).

2.3 MBRA and C. difficile Cultivation Media (See Note 1)

1. BRM (described in ref. [10]): Original bioreactor medium (Table 2).
2. BRM2 (described in ref. [12]): BRM2 is a modification of BRM with 1 g/L taurocholic acid sodium salt replaced by 0.5 g/L bovine bile (Sigma), which is added prior to autoclaving.

Table 2
Recipe for 1 L of BRM

To 975 mL distilled water, add:	
Quantity	**Reagent**
1 g	Tryptone
2 g	Proteose peptone #3
2 g	Yeast extract
100 mg	Arabinogalactan
150 mg	Maltose
150 mg	D-cellobiose
400 mg	Sodium chloride
10 mg	Magnesium sulfate heptahydrate
10 mg	Calcium chloride dihydrate
40 mg	Potassium phosphate dibasic
40 mg	Potassium phosphate monobasic
5 mg	Hemin
2 mL	Tween 80
Adjust to pH 6.8 and autoclave at 121 °C (≥15 psi) for 45 min Post autoclaving, make a filter-sterilized mix of:	
25 mL	Distilled water
1 g	Taurocholic acid, sodium salt (Sigma-Aldrich)
40 mg	D-glucose
200 mg	Inulin
2 g	Sodium bicarbonate
1 mg	Vitamin K_3

3. BDM: A defined medium with nutrient profile similar to BRM2 (Table 3).
4. BHIS Agar (described in ref. [11]): 37 g/L BBL brain heart infusion, 5 g/L yeast extract, 15 g/L Bacto agar.
5. Clindamycin phosphate and vancomycin hydrochloride.

2.4 MBRA Inoculation and Sampling

1. 1.7–2 mL snap cap tubes.
2. 50 mL screw cap conical tubes.
3. 25 mL sterile serological pipettes.
4. Phosphate buffered saline.

Table 3
1 L recipe for BDM

To 950 mL distilled water, add:			
Quantity	**Reagent**	**Quantity**	**Reagent**
600 mg	Ammonium sulfate	170 mg	L-phenylalanine
500 mg	Sodium chloride	180 mg	L-proline
500 mg	Bovine bile	90 mg	L-serine
250 mg	L-alanine	80 mg	L-threonine
190 mg	L-arginine	200 mg	L-valine
210 mg	Glycine	5 mg	Hemin
70 mg	L-histidine	10 mg	Magnesium sulfate heptahydrate
180 mg	L-isoleucine	10 mg	Calcium chloride dihydrate
270 mg	L-leucine	10 mg	Sodium sulfate
240 mg	L-lysine	2 mL	Tween 80
60 mg	L-methionine	1 mL	Trace mineral mix
Autoclave at 121 °C (≥15 psi) for 45 min Post autoclaving, make a filter-sterilized mix of:			
30 mL	Distilled water	40 mg	Potassium phosphate monobasic
200 mg	Inulin	40 mg	Potassium phosphate dibasic
574 mg	L-glutamic acid sodium salt	45 mg	L-tyrosine
32 mg	L-asparagine	250 mg	L-aspartic acid
19 mg	L-cysteine	2 g	Sodium bicarbonate
5 mg	L-glutamine	1 mg	Vitamin K_3
24 mg	L-tryptophan	1 mL	Trace Mineral Mix
Also add 20 mL of autoclaved 2% w/v soluble starch (MP Biomedicals, in distilled water; autoclaved at 121 °C at ≥15 psi for 30 min)			
1 L Trace Mineral Mix. In 998.4 mL distilled water, add			
1.6 mL	Hydrochloric acid	2 mg	Copper(II) chloride dihydrate
2.1 g	Iron sulfate heptahydrate	144 mg	Zinc sulfate heptahydrate
30 mg	Boric acid	36 mg	Sodium molybdate dihydrate
100 mg	Manganese chloride tetrahydrate	25 mg	Sodium metavanadate
190 mg	Cobalt chloride hexahydrate	25 mg	Sodium tungstate dihydrate
24 mg	Nickel(II) chloride hexahydrate	6 mg	Sodium selenite pentahydrate
The stock can be wrapped in foil and stored at 4 °C			

(continued)

Table 3
(continued)

To 950 mL distilled water, add:			
Quantity	**Reagent**	**Quantity**	**Reagent**
1 L Trace Vitamin Mix. In 1 L distilled water, add			
900 mg	Potassium phosphate monobasic	1.55 g	Nicotinic acid
15 mg	Folic acid	120 mg	Calcium pantothenate
60 mg	Pyridoxine hydrochloride	1 mg	Vitamin B12
213 mg	Riboflavin-5-phosphate sodium salt hydrate	12 mg	p-aminobenzoic acid
2 mg	Biotin	5 mg	Lipoic acid
58 mg	Thiamine hydrochloride		
Filter-sterilize. The stock can be wrapped in foil and stored at 4 °C			

5. Laboratory wipes (e.g., 4.5 × 8.5 in Kimwipes).
6. Bleach.
7. 1 and 5 mL syringes.
8. 22 gauge × 3″ hypodermic needles (e.g., Air-Tite Products #N223) and 16 gauge × 1.5″ hypodermic needles.
9. Laboratory centrifuge that can accommodate 50 mL screw cap conical tubes.

2.5 qPCR to Enumerate *C. difficile*

1. 0.1 mm silica/zirconia beads.
2. 1.7–2 mL screw cap tubes.
3. RNAse, DNAse-free molecular grade water or autoclaved ultrapure water (resistance = 18.2 MΩ cm at 25 °C).
4. qPCR primers were previously described [10] and are listed in Table 4. *tcdA* and broad range bacterial 16S rRNA can be used to determine the ratio of toxigenic *C. difficile* DNA to total DNA present in the population. qPCR primers for *thyA* and *thyX* can be used to distinguish *C. difficile* ribotype 027 strains bearing the *thyA* insertion to strains of other *C. difficile* ribotypes carrying the ancestral *thyX* gene.
5. Power SYBR Green PCR Master Mix (Life Technologies) or equivalent.
6. iCycler iQ PCR plates (Bio-Rad) and microseal "C" optical sealing film (Bio-Rad) or equivalent.
7. Laboratory centrifuge that can accommodate 96-well plates.
8. Real-time PCR machine with capability for detecting SYBR green (e.g., Eppendorf Mastercycler ep realplex2).

Table 4
qPCR primer sequences

Target gene	Primer sequences (Forward and Reverse)	Citation
C. difficile tcdA	F: AGC TTT CGC TTT AGG CAG TG R: ATG GCT GGG TTA AGG TGT TG	[10]
Bacterial 16S *rRNA*	F: ACT CCT ACG GGA GGC AGC AG R: ATT ACC GCG GCT GCT GG	[14]
C. difficile thyA	F: GAT GGC CAG CCT GCT CAT ACA ATA R: TGT TTC ATC AGC CCA GCT ATC CCA	[10]
C. difficile thyX	F: CCA GTT GGG ACA GAC GAA AT R: TGA ACA AGC CCT TGA AAT ACC	[10]

3 Methods

3.1 Fecal Sample Preparation for −80 °C Storage

1. Prior to receipt of fecal samples, autoclaved laboratory spatulas, PBS, and cryogenic vials should be placed into the anaerobic chamber to pre-reduce for ≥4 h.
2. Upon receipt, fecal samples should be transferred into the anaerobic chamber along with a portable laboratory scale.
3. Each fecal sample should be stirred with a sterile spatula and divided into aliquots in cryogenic vials at a consistent mass (2–3 g depending upon size of cryogenic vial).
4. A 200 mg aliquot should be removed from the fecal sample, resuspended in 1 mL of PBS, heat-killed at 65 °C for 30 min, and 100 µl spread on TCCFA to verify that the sample is *C. difficile* negative.
5. Samples should be labeled, sealed tightly, removed from the anaerobic chamber, flash frozen with liquid nitrogen, and stored at −80 °C. Samples stored in this way can be used for ≥1 year.

3.2 MBRA Assembly for 24 Reactors (Four MBRA Strips)

1. Use the thread-tapping tool to introduce ¼-28 threads into all ports of the MBRA. Rinse with water to remove plastic particulates (Fig. 1a).
2. Cut twenty-four 25 mm segments from the 1/8″ PTFE tubing (Kinesis Inc.).
3. Securely insert each 25 mm segment of PTFE tubing into a male luer to ¼-28 thread fitting (Fig. 1b).
4. Screw the fitting into the top of the MBRA strip.
5. Add 15 mL of water to each reactor chamber and ensure that the bottom of the PTFE tubing is just touching the surface of the water (Fig. 1c, *see* **Note 2**).

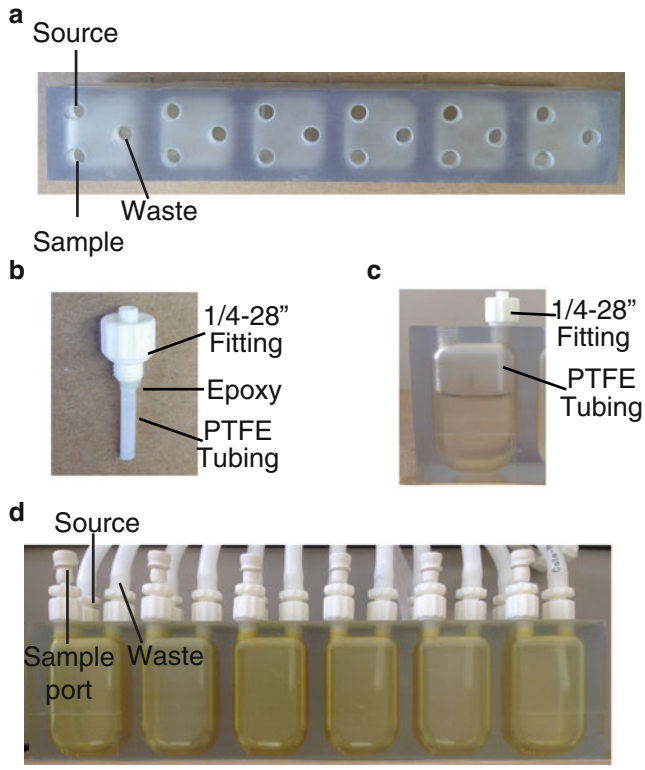

Fig. 1 Initial assembly of MBRA. (**a**) A top-down view of an MBRA strip, indicating the positions at which to secure the source line, waste line, and sample port on one of the reactor chambers. (**b**) An image of a ¼-28″ fitting with inserted PTFE tubing waste line. The position at which epoxy should be applied is also noted. (**c**) Proper length of the PTFE waste line tubing is verified by insertion of a ¼-28″ fitting with inserted waste line into a reactor chamber that is filled with the desired volume of water. (**d**) An assembled MBRA strip, noting the position of the source C-flex tubing, waste C-flex tubing, and sample port with properly folded septum on a single reactor

6. Remove the fittings from the MBRA, maintaining the position of the PTFE tubing.
7. Pour the water out of the MBRA.
8. Using a pipette tip, apply a thin layer of epoxy at the bottom of the ¼-28 thread fitting where it contacts the PTFE tubing to hold in place (Fig. 1b). Take care to avoid excessive addition of epoxy.
9. Allow epoxy to cure, then screw back into the top of the MBRA in the position indicated for the waste line fitting in the MBRA diagram in Fig. 1.
10. Place one 8 × 3 mm micro stir bar in each reactor within the MBRA.
11. Screw additional male ¼-28 thread fittings in the top of the MBRA at the positions for the source line fittings and sample ports.
12. Screw female luer × 1/8″ hose barb adapter fittings into the ¼-28 thread fittings for the waste and source lines.
13. Screw female luer × 3/32″ hose barb adapter fittings into the ¼-28 thread fittings for the sample port.

14. Place rubber septa over 3/32″ hose barb adapters and fold top over to make a flat surface (Fig. 1d).
15. Cut eight pieces of C-flex tubing at each of the following lengths: 14, 15.25, 16.5, 17.75, 19, and 20.25 cm for the source and waste lines.
16. Securely fasten the C-flex tubing over the female luer × 1/8″ hose barb adapter fittings for the source and waste lines (*see* **Note 3**).
17. Insert a male luer with lock ring × 1/8″ hose barb adapter fitting into the free end of the C-flex tubing.
18. Insert female luer × 1/16″ hose barb adapter fittings into each end of the 2-stop Tygon E-Lab Tubing (both 0.89 mm and 1.14 mm, *see* **Note 4**).
19. Screw one end of the tubing with the female luer × 1/16″ hose barb adapter fitting into the male luer with lock ring × 1/8″ hose barb adapter fitting already connected to the C-flex tubing on the MBRA. Take care to connect the appropriate, color-coded 2-stop tubing to the appropriate waste and source lines.
20. Cut twenty-four 5 cm pieces of C-flex tubing for connecting the source lines. Insert male luer with lock ring × 1/8″ hose barb adapter fittings into both ends of the tubing.
21. Connect one end of the 5 cm piece of tubing with male luer with lock ring × 1/8″ hose barb adapter fitting to the unoccupied female luer × 1/16″ hose barb adapter fitting at the end of the 0.89 mm 2-stop E-lab tubing. Repeat for the remaining 23 pieces of tubing.
22. Cut sixteen 5 cm pieces of C-flex tubing. For eight of the tubes, insert a male luer with lock ring × 1/8″ hose barb adapter fitting into both ends of the tubing. For the remaining eight pieces of tubing, insert a male luer with lock ring × 1/8″ hose barb adapter fitting into one end of the tubing and a female luer × 1/8″ hose barb adapter fitting into the other end of the tubing.
23. Join the C-flex tubing for the source lines together with female luer tees (Fig. 2a). The C-flex tubing for the reactors on the rightmost end of the strip should be connected to opposing sides of the tee by screwing the male luer with lock ring × 1/8″ hose barb adapter fittings onto the tee. A 5 cm piece of C-flex tubing with male luer with lock rings × 1/8″ hose barb adapter fittings on both ends should be connected to the tee that is perpendicular to the remaining two outlets. This newly added tubing should be connected to a second tee junction, with the C-flex tubing for the reactor that is in the third position from the right connected to the opposing side of the tee junction. Another 5 cm piece of tubing, with a male luer with lock ring × 1/8″ hose barb adapter fitting at one end and a female luer × 1/8″ hose barb adapter fitting at the other end should be connected to the perpendicular position of the tee. (During operation, this end will be connected to a

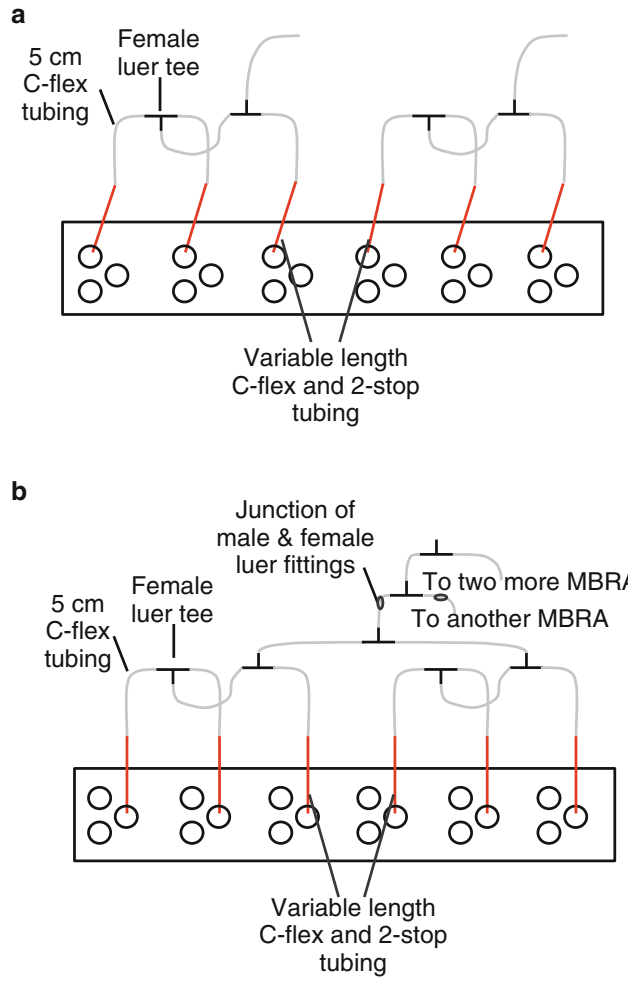

Fig. 2 Connection of source and waste lines. Diagrams of the connections used for the source (**a**) and waste (**b**) lines. More details are described in the text

source bottle for these reactors. During autoclaving, this end should be covered with a piece of aluminum foil to maintain sterility post-autoclaving.) A similar pattern should be used to join the source lines for reactors 4–6 from the right-hand side.

24. Loosely tape the tubing together (grouped in threes) for autoclaving.
25. Begin preparing the waste lines by cutting twenty-four 5 cm pieces of C-flex tubing and inserting the male luer with lock ring × 1/8″ hose barb adapter fittings into both ends the tubing.
26. As for the source lines, connect one of the male luers with lock ring × 1/8″ hose barb adapter fitting to the unoccupied female luer × 1/16″ hose barb adapter fitting at the end of the 1.14 mm 2-stop E-lab tubing. Repeat for remaining 23 pieces of 1.14 mm 2-stop E-lab tubing.

27. Cut twenty 5 cm pieces of C-flex tubing. For 16 pieces of this tubing, insert male luer with lock ring × 1/8″ hose barb adapter fittings into both ends of the tubing. For the remaining four pieces of tubing, insert a male luer with lock ring × 1/8″ hose barb adapter fitting into one end of the tubing and a female luer × 1/8″ hose barb adapter fitting into the opposing end of the tubing.
28. Join the C-flex tubing for the waste lines together with female luer tees (Fig. 2b). This assembly initially proceeds as described for joining the source lines together in **step 23**. However, rather than terminating in a female luer × 1/8″ hose barb adapter fitting as described in **step 23**, the joined sets of three waste line tubing will terminate in a male luer with lock ring × 1/8″ hose barb adapter fitting and be connected together to opposing ends of a tee junction to make a single waste stream exiting from the MBRA. A 5 cm piece of tubing with a male luer adapter with lock ring × 1/8″ hose barb adapter fitting at one end and a female luer × 1/8″ hose barb adaptor fitting at the other end should be connected the tee junction. The free end of the female luer × 1/8″ hose barb adapter fitting should be capped with foil for autoclaving. Assemble remaining three MBRA reactors in this way.
29. Prior to operation, the waste streams from all four reactors will be connected into a single waste stream. Connecting reactors prior to autoclaving makes manipulation awkward. Therefore, we build and autoclave the waste line connector assembly separately. Build the connector assembly by cutting four 3 cm pieces of C-flex tubing and two 5 cm pieces of C-flex tubing and inserting male luer with lock ring × 1/8″ hose barb adapter fittings into both ends of the tubing. Connect two of the 3 cm pieces to opposing ends of a tee junction. Connect one of the 5 cm pieces of C-flex tubing with male luer with lock ring × 1/8″ hose barb adapter fittings at both ends to the perpendicular position on the tee junction. Repeat with a second tee junction, the remaining two 3 cm pieces, and a 5 cm piece of tubing. Connect the 5 cm pieces of tubing present on each of the two tee junctions to opposing sides of a new tee junction. Cut a 25–50 cm piece of C-flex tubing (size depends upon configuration in your anaerobic chamber). Place a male luer with lock ring × 1/8″ hose barb adapter fitting on one end and a female luer × 1/8″ hose barb adapter fitting on the opposing end. Connect the 25–50 cm piece of C-flex tubing to the perpendicular side of the tee junction. The terminal female luer fitting should be covered with foil and the tubing should be grouped together and loosely taped for autoclaving.
30. If your anaerobic chamber setup contains pass-through ports, such as the butyl rubber stoppers present on the Coy Vinyl anaerobic chamber, a ¼″ hole can be drilled through the center

of the butyl stopper. The stopper can be cut to allow insertion of the C-flex tubing, followed by resealing with epoxy, which is reinforced with additional epoxy following autoclaving. If your anaerobic chamber does not have pass-through ports, the waste can be collected within a waste container in the anaerobic chamber and removed daily (*see* **Note 5**).

31. Assemble a waste collection bottle by loosely securing an Omnifit Q-series two-hole bottle cap to the top of a 2 L bottle. Screw male ¼-28 thread fittings into both of the holes of the cap. Screw on female luers with 1/8″ hose barb adapters into both male ¼-28 thread fittings. Cut a 5 cm piece of C-flex tubing and connect to one of the 1/8″ hose barb adapters on the waste collection bottle. Insert a male luer with lock ring 1/8″ hose barb adapter at the opposing end of the 5 cm piece of tubing and cover with foil (This piece of tubing will serve as your air vent; contamination will be prevented by connecting a 0.22 μm syringe filter following autoclaving; *see* **Note 6**.). Cut a 20 cm piece of C-flex tubing and connect to the second 1/8″ hose barb adapter on the waste collection bottle. Place a male luer with lock ring × 1/8″ hose barb adapter in the opposite end of the tubing and cover with foil for autoclaving. This fitting will be connected to the waste line leaving the anaerobic chamber following autoclaving.
32. Autoclave reactors at 121 °C, ≥15 psi for 45 min. Use slow exhaust program typically used for liquids.
33. After reactors cool, tighten fittings and place in anaerobic chamber for at least 72 h prior to use.

3.2.1 MBRA Source Bottle Assembly and Media Preparation

1. Assemble 24 source bottles by placing an Omnifit Q-series two-hole bottle cap on each 1 L media bottle. Opposite the full length piece of PTFE tubing that comes inserted in each cap, insert a ¼-28 mm thread to barbed male adaptor (3.2 mm) and screw in securely. Cut a 20 cm piece of C-flex tubing and place on the ¼-28 mm thread to barbed male adaptor. At the other end of the 20 cm tubing, insert a male luer with lock ring 1/8″ hose barb adaptor fitting. Cover with foil. In the second hole on the Omnifit cap, insert a male luer to ¼-28 thread fitting. Screw a female luer to 1/8″ hose barb adapter fitting on to the male luer fitting. Cut a 5 cm piece of C-flex tubing and place over the 1/8″ hose barb of the female luer fitting. At the other end of the 5 cm tubing, insert a male luer with lock ring 1/8″ hose barb adaptor fitting. Cover with foil. (This will be your vent line during operation and will be coupled to a 0.22 μm syringe filter to prevent contamination.)
2. Media should be prepared as described in the materials section (*see* **Note 7**), with media dispensed into source bottles prior to autoclaving. Caps should be placed loosely on the source bottles

and autoclaved on the slow exhaust cycle for 45 min at 121 °C (≥15 psi). Media should be placed in the anaerobic chamber for at least 72 h prior to use to ensure sterility and anaerobicity.

3. Only eight source bottles are needed at one time for operation of 24 MBRA. We make all 24 L at one time to ensure consistency and place bottles into the chamber ≥72 h prior to use. Each liter of medium lasts approximately 1 week with the flow conditions described below.

3.3 MBRA Setup

1. MBRA should be aligned in holders on the magnetic stir plate over the indicated stirring positions. Proper alignment can be verified by setting the stir plate controller to the maximum rpm and verifying that the magnetic stir bars are turning.
2. 2-stop lab tubing for the source and waste should be properly fitted into the clamps on the peristaltic pump. If tubing is placed into the peristaltic pump clamps <24 h prior to intended use, clamps should be locked in place. Otherwise, delay locking clamps until <24 h before use because this reduces the chance of tubing compression.
3. Connect the terminal piece of C-flex tubing on the waste lines from each MBRA strip to the waste line connector assembly by joining male and female luer connections (*see* **Note 8**). Connect tubing to the waste bottle (s).
4. Connect the terminal pieces C-flex tubing on the source lines to the 20 cm piece of tubing on each source bottle.
5. Place a 0.22 μm syringe filter on the 5 cm vent line for each source bottle and on the vent line for the waste bottle.
6. Initiate flow at a modest flow speed (1 mL/min on source pump and 2 mL/min on waste pump) to begin filling the reactors.
7. As reactors begin to fill, check that all reactors reach the same volume and that media exits the reactors into the waste line. If any individual reactors do not fill, tighten any loose fittings and check for problems with individual source lines and replace with prepared extra lines if necessary. If an entire set of three reactors does not fill, check for problems with the source bottle by tightening fittings. If problem is not resolved, replace with a new source bottle, if available. If level of medium rises above the interior waste line in the reactors, check the fittings and exterior waste line for problems and replace if necessary.
8. Once each reactor has filled, reduce the pump settings to your desired flow rate (*see* **Note 9**) and then turn off the pumps.
9. Leave medium in the reactors and waste bottle(s) for 16–24 h prior to inoculation to ensure sterility was maintained.

3.4 MBRA Inoculation

1. Place autoclaved laboratory spatulas, PBS, 50 mL conical tubes, 25 mL sterile serological pipettes, 5 mL syringes, and 16 gauge needles in anaerobic chamber to pre-reduce for ≥4 h.
2. Determine the amount of fecal material that is needed to inoculate the desired number of reactors. Each reactor receives 3.8 mL of supernatant from a 25% m/v fecal slurry. For 24 reactors, a total of 144 mL of 25% m/v fecal slurry should yield sufficient supernatant for inoculation.
3. Transfer frozen fecal samples to the anaerobic chamber and thaw for 15 min.
4. Use a sterile laboratory spatula to transfer fecal material from cryogenic vials to 50 mL conical tubes (12 g total mass). Add 36 mL of anaerobic PBS and seal conical tube tightly. Repeat for two more tubes.
5. Remove conical tubes from chamber, vortex for 5 min, then centrifuge at low speed (201 × *g*) for 5 min to settle particulates.
6. While samples are centrifuging, sterilize the septa on top of each reactor with a laboratory wipe wetted with 10% bleach. Leave in place for 10 min.
7. Return centrifuged samples to anaerobic chamber.
8. Using sterile serological pipettes, transfer supernatants (~35 mL) to new 50 mL conical tubes.
9. Load 3.8 mL fecal slurry supernatant into 5 mL syringes with 16 gauge 1.5″ needles.
10. Quickly remove laboratory wipe, fully insert the needle through the center of the septum, and inject fecal slurry into the reactor.
11. If desired, save a 1 mL aliquot of fecal slurry for analysis (*see* **Note 10**). Transfer 1 mL to 1.7 mL tube. Centrifuge at 20,000 × *g* for 1 min. Transfer the supernatant to new 1.7 mL tube and store both tubes at −80 °C.
12. Allow fecal communities to begin growing in batch mode for 16–18 h before initiating flow to reactors. (Ensure that magnetic stir plate control is on and at maximum rpm). If desired, samples can be removed and processed as described in **step 15** prior to initiation of flow.
13. After 16–18, restart peristaltic pumps to initiate flow to reactors.

3.5 MBRA Operation and C. difficile Invasion

1. Once communities have been established with continuous flow for at least 24 h, communities can be disrupted by treatment with antibiotics for 4 days (*see* **Note 11**).
2. Prepare a 25 mg/mL stock of clindamycin phosphate in water and filter-sterilize. Sterilize septa with laboratory wipes and 10% bleach as described above. Inject each reactor to be disrupted with 150 µl of 25 mg/mL clindamycin using a 1 mL syringe and 22 gauge 3″ needle (250 µg/mL final concentration, *see* **Note 12**).

Dose each source bottle with appropriate volume of 25 mg/mL clindamycin to achieve 250 μg/mL final concentration.

3. If you are interested in comparing the composition of your communities before and after treatment (*see* **Note 10**), you should collect a 1 mL sample from each reactor prior to administration of clindamycin. To collect samples, sterilize the septa with bleach and laboratory wipes as described above. Insert a 22 gauge 3″ needle connected to a 1 mL syringe through the septum of each reactor. Carefully remove 1 mL of sample. Transfer to 1.7 mL tube and seal. Remove from the anaerobic chamber and centrifuge at 20,000 × *g* for 1 min to pellet cells. Transfer supernatants to new tube and store pellets and supernatants at −80 °C.
4. Samples can be removed from reactors daily, if desired, during antibiotic treatment and processed as described above.
5. *C. difficile* will be added to reactors ~24 h following the cessation of antibiotic treatment. Therefore, it is essential to begin preparing *C. difficile* cultures in advance. If spores are to be used, they should be prepared and titered prior to use using appropriate protocols. If vegetative cultures will be used, they should be propagated on BHIS agar two days prior to use. At this time, 5 mL aliquots of BRM2 medium in 15 mL conical tubes should be prepared and pre-reduced. Following propagation on BHIS, *C. difficile* strains should be inoculated into 5 mL aliquots of BRM2 (1 aliquot per strain) and inoculated anaerobically at 37 °C overnight. Following overnight incubation, *C. difficile* strains should be diluted 1:20 in fresh BRM2 and their growth should be monitored periodically until cells reach an optical density at 600 nm of 0.08–0.15. At this point, *C. difficile* cells should be diluted 1:10 into a fresh aliquot of BRM2 and used to inoculate reactors. If you are studying competition between two strains of *C. difficile*, they can be mixed at this dilution step.
6. After 4 days of clindamycin treatment, disconnect old source bottles and replace with fresh source medium lacking antibiotic (*see* **Note 13**).
7. If you will be monitoring *C. difficile* abundance by serial dilution and selective plating, transfer appropriate selective agar (e.g., TCCFA with additional antibiotics), pipette tips, 1.7 mL tubes, and aliquots of BRM2 agar to chamber to pre-reduce (*see* **Note 14**).
8. 16–24 h after replacing clindamycin-containing source medium with fresh medium, sample all reactors. This sample will be used as a community background (negative) control for *C. difficile* in qPCR assays.
9. If using vegetative cells, subculture *C. difficile* as described above in **step 5**. If using spores, *C. difficile* can be added at any time following sampling.

10. Inoculate spores or subcultured *C. difficile* cells into reactors through sterilized septa with 22 gauge 3″ needles and 1 mL syringes. Spores should be added to achieve a final concentration of 10^6 mL in reactors. Addition of 150 μl of *C. difficile* vegetative cells as described above should result in a final density in reactors of 10^4–10^5 cells/mL.
11. After 10–15 min to allow mixing of *C. difficile*, remove 1 mL samples from reactors and transfer to 1.7 mL tubes as described above.
12. If *C. difficile* is to be enumerated by selective plating, remove a 100 μl aliquot for spot plating and transfer to a new tube.
13. Remove remainder of sample in 1.7 mL tube, centrifuge and process as described in **step 3**.
14. If enumerating by selective plating, serially dilute *C. difficile* sample to 10^{-3} and spot plate 10 μl of undiluted, 10^{-1}, 10^{-2} and 10^{-3} dilutions on selective agar. Incubate plates at 37 °C anaerobically for 16–24 h and count colonies.
15. *C. difficile* proliferation can be monitored over time by sampling followed by selective plating as described in **steps 12** and **14** and/or by qPCR with *C. difficile* primers as described below (*see* **Note 15**).
16. Source bottles should be replaced with new source bottles containing fresh medium when remaining volume in the current source bottle is ~100 mL.
17. At the end of the run, the source medium can be replaced with source media bottles containing a 20% bleach solution to sterilize the MBRAs. 20% bleach should be run through the reactors at moderate pump setting (1 mL/min) and left in contact with MBRA for 30–60 min (*see* **Note 16**). Remove reactors from chamber, disassemble, empty, and rinse. Fittings and MBRA strips can be reused until visible signs of wear and tear are detected.

3.6 Alternative Bioreactor Assembly

1. We have made small volume bioreactors using 100–500 mL GL45 thread media bottles fitted with Omnifit Q-series two hole bottle caps. The volume within the reactor is fixed by the length of the PTFE waste tubing. Reactors can be stirred with a standard magnetic stir plate at a low setting and small laboratory magnetic bars (22 × 8 mm). Media can be delivered and removed with a smaller peristaltic pump. Appropriate pump settings should be determined by measuring the amount of water transferred over a fixed period of time within the setup. Source and waste bottle assembly follow a similar pattern to that described for the MBRAs, with the 2-stop lab tubing connected to C-flex tubing by luer fittings. We have not found a needle of appropriate length to sample these reactors. Therefore, the cap must be removed each time a sample is desired.
2. Other aspects of bioreactor preparation and operation described above can be used with this simplified setup.

3.7 Quantification of C. difficile Levels by qPCR

1. *C. difficile* levels can be quantified directly from bead beaten cell extracts.
2. Prepare tubes for bead beating by adding ~700 mg of 0.1 mm zirconia/silica bead to a 1.7 mL screw cap tube. Add 100 µl of molecular grade water. Leave lid lightly fastened on top. Autoclave for 20 min at 121 °C (≥15 psi). Cool and tighten cap prior to use.
3. Bead beat the cells by resuspending the cell pellet from 1 mL of cells in 500 µl molecular grade water. Transfer to bead beating tube and tighten cap. Homogenize with bead beater at maximum speed for 2 min. Centrifuge at 8000 × *g* for 1 min to pellet beads. Transfer supernatant to new tube and store at −20 °C until use.
4. For toxigenic *C. difficile*, the toxin A gene (*tcdA*) primers described in Subheading 2 can be used to quantify the levels of *C. difficile*. For absolute quantification, prepare *C. difficile* standards from a known quantity of *C. difficile* cells. To make these standards, grow your *C. difficile* strain overnight in 5 mL of BRM2. Remove a 100 µl aliquot, serially dilute and spot 10 µl of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution on TCCFA, BHIS or BRM2 agar plate. Incubate plate anaerobically at 37 °C overnight to determine CFU/mL. Transfer a 1 mL aliquot from overnight to a 1.7 mL tube, centrifuge at 20,000 × *g* from 1 min to pellet, remove supernatant, and store pellet at −80 °C until ready for bead beating, which should be performed as described above. Prepare bead beaten extracts from your sample collected before addition of *C. difficile*. Pool 50 µl from each reactor to act as the background community control. Using the CFU/mL data obtained from plating, serially dilute your *C. difficile* standard in to 10^7 CFU equivalents into background community control extract in a total volume of 500 µl. For example, if plating data indicated that *C. difficile* was present at 10^8 CFU/mL (typical concentration for overnight growth in BRM), dilute 50 µl of *C. difficile* bead beaten extract into 450 µl of background community extract. Perform tenfold serial dilutions into background community control DNA (200 µl total volume) to 10^3 CFU/mL. Use 4 µl of each standard, 10^7–10^3, to establish a *C. difficile* standard curve for calibration of qPCR data. Use 4 µl of the background community control as a negative control for *C. difficile*.
5. To perform qPCR on samples, transfer 4 µl of bead beaten cell extract from each sample to the wells of a 96-well optical plate. (All reactions should be done in triplicate.) Transfer 4 µl of each standard as well as negative control to the 96-well optical plate. Prepare qPCR master mix. The 1× concentration for master mix is 12.5 µl Power SYBR Green PCR Master mix, 0.25 µl 5 µM *tcdA* forward primer, 0.25 µl 5 µM *tcdA* reverse primer, and 8 µl molecular grade water. Transfer 21 µl of master mix to each

reaction. Cover plate with optical film and centrifuge briefly at low speed to remove any air bubbles. Amplify in qPCR machine with the following cycle: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min (fluorescence measured at the end of each 60 °C cycle). A 20-min melting curve can be performed from 60 to 95 °C to ensure that the amplified product is consistent with the *tcdA* product amplified in the standards.

6. Analyze the qPCR data by examining the mean and standard deviations of the cycle thresholds (C_T) values for each replicate. If the standard deviation of the C_T values for any of the replicates are larger than 1.5, consider repeating. Plot a standard curve from the controls, using the $\log_{10}$ value of the CFU/mL on the *y*-axis and the average C_T value on the x-axis. Determine the equation and R^2 for the line; it is not necessary to force the line through 0. Use the equation from your standard curve and the C_T values to determine the CFU/mL equivalents from your samples. For samples with C_T values below the range of your standard curve, report the CFU/mL levels as below the limit of detection. If you encounter samples with C_T values above your standard curve, repeat the qPCR on a 1:10 or 1:100 dilution of your sample.
7. As a further normalization for qPCR, the total 16S rRNA gene signal can be determined using the broad-range qPCR primers described in Materials. Because the primers have a broad-range for 16S rRNA genes, care should be taken to avoid contamination with source of bacterial DNA. Use fresh molecular grade water, sterile tubes, and barrier tips for dilutions and qPCR setup. A standard curve can be created from community DNA. In our experience, the MBRA communities in BRM2 support a density of 10^8 cells/mL. However, this can be verified for your specific community by serial dilution and spot plating of MBRA samples on BRM2 agar if desired. Serially dilute community cell extracts from 10^6–10^2 CFU/mL equivalents in molecular grade water in a total volume of 100 μl. Use 4 μl of each standard (10^6–10^2) as well as a molecular grade water as a negative control. Dilute each sample 1:100 in molecular grade water. Transfer 4 μl of each 1:100 dilution, each standard and negative control to qPCR plate. (Set up reactions in triplicate.) Prepare qPCR master mix. 1× concentration for master mix is: 12.5 μl Power SYBR Green PCR Master mix, 0.25 μl 5 μM 16S *rRNA* forward primer, 0.25 μl 5 μM 16S *rRNA* reverse primer, and 8 μl molecular grade water. Transfer 21 μl of master mix to each reaction. Cover plate with optical film and centrifuge briefly at low speed to remove any air bubbles. Amplify in qPCR machine with the cycle described above for *tcdA*. A 20 min melting curve should be performed from 60 to 95 °C to ensure that the amplified product is consistent with the 16S *rRNA* product amplified in the standards.

8. Analyze the qPCR data by examining the mean and standard deviations of the C_T values for each replicate. If the standard deviation of the C_T values for any of the replicates are larger than 1.5, consider repeating. Plot a standard curve from the controls, using the $\log_{10}$ value of the CFU/mL on the *y*-axis and the average C_T value on the *x*-axis. Determine the equation and R^2 for the line; it is not necessary to force the line through 0. You will most likely observe a signal from the negative control. As long as this signal is at least 2 C_T units lower than your 10^2 control you should not be concerned. Use the equation from your standard curve and the C_T values to determine the CFU/mL equivalents from your samples. If you encounter samples with C_T values above your standard curve, repeat the qPCR on a 1:1000 or 1:10,000 dilutions of your sample.
9. Normalize your calculated *tcdA* CFU/mL across samples based upon the total bacterial DNA signals. In our experience, C_T values for total bacterial DNA vary by less than two across samples, so the impact is typically modest.
10. If you are using primers to compare levels of two different *C. difficile* populations, such as the *thyA*-containing ribotype 027 strains and strains of other ribotypes that contain the *thyX* gene, use these minor variations to the protocol described below. Previous qPCR experiments with the *thyA* and *thyX* described in Materials have demonstrated that the efficiency of the two primer sets, calculated with the method described by Pfaffl [13] differ by <5%, so direct comparisons of the C_T values detected can be made without comparison to a standard curve. Samples are bead beaten as described above and are diluted 1:10 prior to use of 1 μl as template in qPCR reactions. (Reactions are still performed in triplicate.) The 1× master mix has higher volumes of water (11 μl) to compensate for the decreased template and higher concentrations of primers (100 μM rather than 5 μM). A 1× master mix contains: 12.5 μl Power SYBR Green PCR Master mix, 0.25 μl 100 μM *thyA* or *thyX* forward primer, 0.25 μl 100 μM *thyA* or *thyX* reverse primer, and 11 μl molecular grade water. *thyA* and *thyX* are amplified in separate PCR reactions using the cycling parameters described above.
11. Analyze qPCR results. The ratio of *thyA* to *thyX* is calculated with the equation $2^{(C_T thyX - C_T thyA)}$. The competitive index for a specific day is determined by dividing the ratio on a specific day to the ratio observed on day 0, just after addition of *C. difficile*.
12. Competitions between other strains of *C. difficile* can be monitored by qPCR as long as you have primers that distinguish each strain being competed.

4 Notes

1. Unless otherwise noted, reagent-grade chemicals from any manufacturer can be used in media.
2. The length of the PTFE tubing can be adjusted to change the volume of the reactor vessel. Shortening the line can increase the retention volume whereas lengthening the line can decrease the retention volume. We have used retention volumes as small as 5.2 mL and as large as 20 mL. To avoid contamination of the source medium, leave a headspace volume equivalent to 5 mL of medium.
3. To minimize the amount of source and waste line tubing, C-flex tubing length decreases with the reactors' proximity to the source or waste peristaltic pump. That is, if the peristaltic pump loaded with waste lines is on the right hand side, then the 14 cm C-flex tubing for the waste line will be on the right-most reactor within the MBRA strip. By necessity, this will be the reactor that is most distant from the peristaltic pump loaded with source lines and so the 20.25 cm C-flex tubing for the source line will be on the right-most reactor.
4. The interior diameter of the 2-stop Tygon E-lab tubing is smaller than the 1/16″ female luer fitting hose barb adapter fitting (~1.6 mm). With some effort, both the 0.89 and 1.14 mm tubing can be placed over the hose barb adapter. Placing the fitting over the hose barb adapter is facilitated by heating the ends of the 2-stop Tygon E-lab tubing in hot water (>65 °C) just prior to inserting the fitting into the tubing. During MBRA setup in the anaerobic chamber, you will occasionally encounter problems with a pinched piece of 2-Stop tubing impeding media flow. It is advisable to prepare two extra pieces of 2-stop Tygon E-lab tubing of each size (0.89 and 1.14 mm) with female luer to 1/16″ hose barb adapter fittings on each end. The fittings on the ends of these pieces of tubing can be capped with foil, autoclaved, placed in the anaerobic chamber, and used to replace any damaged tubing discovered during MBRA setup.
5. Many communities cultivated in MBRA produce hydrogen sulfide, which poisons the palladium catalysts used for catalyzing the reaction between atmospheric hydrogen and trace amounts of oxygen. Poisoning of the catalysts with hydrogen sulfide is irreversible and can quickly diminish the life of palladium catalysts, as well as potentially impacting other electronics within the chamber. Further, some oxygen sensors (e.g., Coy Laboratories Anaerobic Monitor Model 12) use palladium to sense oxygen levels and can also be poisoned by hydrogen sulfide in the chamber. Removal of the waste from the chamber through a pass-through port reduces the level of atmospheric hydrogen sulfide that accumulates and works in

conjunction with hydrogen sulfide mitigation systems (e.g., Hydrogen Sulfide Removal Column from Coy Laboratories; Anatox Activated Charcoal from Shel lab) to prolong the life of palladium catalysts and equipment.

6. Depending upon space considerations, multiple waste bottles can be connected in series. In this case, the first waste bottle should be a 1 L bottle with an Omnifit two-hole cap as described above. In place of the vent line, a ¼-28 mm thread to barbed male adaptor (3.2 mm) should be screwed in securely. On the inside of the Omnifit cap opposite this fitting, a ~25 cm piece of 1/8″ PTFE tubing should be inserted. Insertion of this tubing should leave ~100 mL retention volume. A 20–30 cm piece of C-flex tubing can be placed over the ¼-28 mm thread to barbed male adapter fitting and connected to a second 2 L waste bottle. The 2 L waste bottle should have an Omnifit two-hole cap, with male luer to ¼-28 thread adapter fittings and female luer with 1/8″ hose barb adapter fittings screwed into both holes. The 20–30 cm piece of C-flex tubing originating from the first waste bottle can be connected directly to the hose barb of one of the female luers. The second female luer can be connected to a 5 cm piece of C-flex tubing and male luer with lock ring 1/8″ hose barb adapter fitting to create a vent line. The tubing for the waste bottles should be adjusted in length to ensure that there are no kinks or curls in the line that would impede the flow of media.
7. BRM2 medium is appropriate for most *C. difficile* MBRA experiments and is the most cost-effective medium. BRM contains taurocholate rather than the bovine bile found in BRM2, making this medium more expensive as well as leading to communities with abundant *Bilophila* species that are not observed in BRM2 [10, 12]. BDM attempts to simulate the nutrient composition of BRM2 using defined media. Although microbial communities cultivated in BDM are different than those cultivated in BRM2 (Auchtung and Britton, unpublished results), *C. difficile* is still capable of proliferating in these communities following disruption with clindamycin.
8. As long as connections are made quickly after removal of foil and are not touched against gloves or surfaces, there is a minimal chance for contamination. If fittings become dislodged or touch surfaces, soak them in 10% bleach for 10 min prior to reconnection.
9. For the Watson-Marlowe pumps described above, a flow setting of 1 on the source pump will give a flow rate of 1.875 mL/h, resulting in an 8 h retention time. This setting is what we have typically used for our *C. difficile* invasion experiments. However, we have also successfully implemented this model with a pump setting of 0.5 (0.94 mL/h; 16 h retention time), although *C. difficile* titers are ~1 log lower in these communities (Auchtung

and Britton, unpublished results). We typically use a flow setting on the waste pump that is 2× the flow setting on the source pump to prevent clogging in the waste lines.

10. Microbial communities can be analyzed by amplification of the 16S rRNA gene as previously described [10, 12]. An alternative protocol for microbial community characterization by selective plating has been previously described [3]. Metabolites present in supernatants can be analyzed using protocols appropriate for the metabolite of interest.
11. We typically wait for 24–48 h before disrupting communities with clindamycin treatment, although we have also had success disrupting communities that have been established for 14 days prior to antibiotic treatment and challenge with *C. difficile* (Auchtung and Britton, unpublished results). Previously, we published a regimen of twice daily dosing of reactors with clindamycin (final concentration 500 μg/mL) for 4 days [10]. However, we have found that adding clindamycin directly to reactors once (final concentration 250 μg/mL) followed by addition to the source medium (250 μg/mL) for 3 or 4 days also leads to community disruption and *C. difficile* invasion (Auchtung and Britton, unpublished results). Because this less invasive approach to antibiotic administration successfully disrupts communities, it is now the approach that we use in our experiments.
12. We would recommend leaving a subset of reactors untreated as a control while initially establishing your MBRA model. Once MBRA operation conditions have been robustly established, then all reactor communities could be disrupted with clindamycin if the purpose of your experiment is to study *C. difficile* physiology when proliferating in a disrupted community.
13. When switching source media bottles, disconnect the fitting on the old source bottle from the source line on the reactors and quickly connect the fitting on the new source bottle. Take care to avoid contaminating any of the fittings. Transfer the 0.22 μm filter from the vent line on the old source bottle to the new source bottle. Pulse the source peristaltic pump for 2–3 s at maximum speed to ensure that source medium is being drawn up the interior PTFE tubing line within the source bottle.
14. With a large number of reactors, it is easier to sample reactors into deep well 96-well plates rather than individual 1.7 mL tubes. We also perform serial dilutions in 96-well assay plates using a multichannel pipettor.
15. In our invaded communities, *C. difficile* typically persist at 10^5–10^6 cells/mL (e.g., [10]). In untreated communities, *C. difficile* normally washes out to undetectable levels by days 2–4 following inoculation (e.g., [10]). If desired, *C. difficile* proliferation within a community can be disrupted by treatment

with vancomycin. Vancomycin treatment (5 μg/mL final concentration) for 3 days (administered as described for clindamycin treatment) is sufficient to kill all vegetative cells and will also lead to a slight decrease in *C. difficile* spores due to washout of reactors. Cessation of vancomycin treatment can lead to germination of remaining spores, thereby simulating some aspects of a recurrent *C. difficile* infection (Auchtung and Britton, unpublished results).

16. 20% bleach contact times longer than 1 h lead to discoloration of MBRA.

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