Journal of Visualized Experiments

Deployment of the In Situ Chemotaxis Assay (ISCA) to examine microbial behavior in aquatic ecosystems

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61062R1
Full Title:	Deployment of the In Situ Chemotaxis Assay (ISCA) to examine microbial behavior in aquatic ecosystems
Section/Category:	JoVE Biology
Keywords:	Marine microbiology; Microfluidics; Microbial ecology; Chemotaxis; Behavioral Assay; In Situ
Corresponding Author:	Estelle Emilie Clerc Eidgenossische Technische Hochschule Zurich Departement Bau Umwelt und Geomatik Zürich, ZH SWITZERLAND
Corresponding Author's Institution:	Eidgenossische Technische Hochschule Zurich Departement Bau Umwelt und Geomatik
Corresponding Author E-Mail:	eclerc@ethz.ch
Order of Authors:	Estelle Emilie Clerc
	Jean-Baptiste Raina
	Bennett S. Lambert
	Justin Seymour
	Roman Stocker
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Zürich, ZH, Switzerland

Thursday, January 30, 2020

Dear Dr. Steindel,

We have fully revised our manuscript: “Deployment of the *In Situ* Chemotaxis Assay (ISCA) to examine microbial behavior in aquatic ecosystems” in light of the reviewers’ recommendations.

We have also attached our point by point response to their comments.

We certify that this manuscript has not been previously published and has not been submitted for publication elsewhere while under consideration. We declare no Conflict of Interest.

Thank you very much for handling the manuscript and for engaging with us. We remain at your disposal for any additional clarification and in the meantime, we send you our best regards.

Yours sincerely on behalf of the authors,

Estelle E. Clerc

TITLE:

In Situ Chemotaxis Assay to Examine Microbial Behavior in Aquatic Ecosystems

AUTHORS AND AFFILIATIONS:

Estelle E. Clerc[1], Jean-Baptiste Raina[2], Bennett S. Lambert[3], Justin Seymour[2], Roman Stocker[1]

[1]Institute of Environmental Engineering, Department of Civil, Environmental and Geomatic Engineering, ETH Zürich, Zürich, Switzerland
[2]Climate Change Cluster, University of Technology Sydney, NSW, Australia
[3]School of Oceanography, University of Washington, Seattle, WA, USA

Corresponding Authors:

Roman Stocker (romanstocker@ethz.ch)
Jean-Baptiste Raina (Jean-Baptiste.Raina@uts.edu.au)

Email Addresses of Co-authors:

Estelle E. Clerc (eclerc@ethz.ch)
Jean-Baptiste Raina (Jean-Baptiste.Raina@uts.edu.au)
Bennett Lambert (Lambertb@uw.edu)
Justin Seymour (Justin.Seymour@uts.edu.au)
Roman Stocker (romanstocker@ethz.ch)

KEYWORDS:

microfluidics, chemotaxis, in situ, marine microbiology, microbial ecology, behavioral assay

SUMMARY:

Presented here is the protocol for an in situ chemotaxis assay, a recently developed microfluidic device that enables studies of microbial behavior directly in the environment.

ABSTRACT:

Microbial behaviors, such as motility and chemotaxis (the ability of a cell to alter its movement in response to a chemical gradient), are widespread across the bacterial and archaeal domains. Chemotaxis can result in substantial resource acquisition advantages in heterogeneous environments. It also plays a crucial role in symbiotic interactions, disease, and global processes, such as biogeochemical cycling. However, current techniques restrict chemotaxis research to the laboratory and are not easily applicable in the field. Presented here is a step-by-step protocol for the assembly and deployment of an in situ chemotaxis assay (ISCA), a device that enables robust interrogation of microbial chemotaxis directly in the natural environment. The ISCA is a microfluidic device consisting of a 20 well array, in which chemicals of interest can be loaded. Once deployed in aqueous environments, chemicals diffuse out of the wells, creating concentration gradients that microbes sense and respond to by swimming into the wells via chemotaxis. The well contents can then be sampled and used to (1) quantify strength of the chemotactic responses to specific compounds through flow cytometry, (2) isolate and culture responsive microorganisms, and (3) characterize the identity and genomic potential of the

responding populations through molecular techniques. The ISCA is a flexible platform that can be deployed in any system with an aqueous phase, including marine, freshwater, and soil environments.

INTRODUCTION:

Diverse microorganisms use motility and chemotaxis to exploit patchy nutrient environments, find hosts, or avoid deleterious conditions[1–3]. These microbial behaviours can in turn influence rates of chemical transformation[4] and promote symbiotic partnerships across terrestrial, freshwater, and marine ecosystems[2,5].

Chemotaxis has been extensively studied in laboratory conditions for the past 60 years[6]. The first quantitative method to study chemotaxis, the capillary assay, involves a capillary tube filled with a putative chemoattractant immersed in a suspension of bacteria[6]. Diffusion of the chemical out of the tube creates a chemical gradient, and chemotactic bacteria respond to this gradient by migrating into the tube[7]. Since the development of the capillary assay, still widely used today, many other techniques have been developed to study chemotaxis under increasingly controlled physical/chemical conditions, with the most recent involving the use of microfluidics[8–10].

Microfluidics, together with high-speed video microscopy, enables tracking of the behavior of single cells in response to carefully controlled gradients. Although these techniques have vastly improved the understanding of chemotaxis, they have been restricted to laboratory use and do not translate easily to field deployment in environmental systems. As a consequence, the capacity of natural communities of bacteria to use chemotaxis within natural ecosystems has not been examined; thus, current understanding of the potential ecological importance of chemotaxis is biased toward artificial laboratory conditions and a limited number of laboratory-cultured bacterial isolates. The recently developed ISCA overcomes these limitations[11].

The ISCA builds on the general principle of the capillary assay; however, it makes use of modern microfabrication techniques to deliver a highly replicated, easily deployable experimental platform for the quantification of chemotaxis toward compounds of interest in the natural environment. It also allows identification and characterization of chemotactic microorganisms by direct isolation or molecular techniques. While the first working device was self-fabricated and constructed of glass and PDMS[11], the latest injection-molded version is composed of polycarbonate, using a highly standardized fabrication procedure (for interest in the latest version of the device, the corresponding authors can be contacted).

The ISCA is credit card-sized and consists of 20 wells distributed in a 5 well x 4 well array, each linked to the external aquatic environment by a small port (800 µm in diameter; **Figure 1**). Putative chemoattractants loaded into the wells diffuse into the environment via the port, and chemotactic microbes respond by swimming through the port into the well. As many factors can influence the outcome of an ISCA experiment in the natural environment, this step-by-step protocol will help new users overcome potential hurdles and facilitate effective deployment.

PROTOCOL:

It is recommended to execute section 1 prior to field experiments to optimize results.

1. Laboratory optimization

NOTE: The volumes described in the optimization procedure are sufficient for a single ISCA (composed of 20 wells).

1.1. Preparation of the chemical of interest

NOTE: The optimal concentration for each chemoattractant often needs to be determined under laboratory conditions prior to field deployments. The chemical concentration field will decrease in magnitude with distance from the source (ISCA well), which means that the concentration experienced by microorganisms in the environment will be lower than that present inside the device. The optimal concentration to use in the ISCA well depends on the diffusion rate of the chemoattractant. If the concentration in the well is too low (close to the detection limit of the microbes), no positive chemotaxis will be observed. Conversely, if the concentration in the well is too high, the concentration will also be high in the immediate environment and microbial accumulation will occur above the ISCA wells rather than inside. Therefore, dilution series should be carried out in laboratory conditions for each compound in order to determine the optimal concentration for field deployments. Ideally, a concentration range should also be tested in the field to confirm laboratory results.

1.1.1. Prepare 2.5 L of a suitable medium containing the appropriate salt concentration (e.g., phosphate-buffered saline [PBS] or artificial seawater). Filter the medium with a 0.2 µm filter using a peristaltic or vacuum pump and autoclave.

1.1.2. Prepare a 10 mM solution of chemoattractant in 1 mL of the sterile medium. Filter the chemoattractant solution with a 0.2 µm syringe filter to remove particles and potential contaminants.

NOTE: Ideally, no organic compound other than the chemoattractant should be present in the final solution.

1.2. Concentration range by dilution series

1.2.1. Dilute the filtered chemoattractant stock solution in series, ranging (for example) from 10 mM to 100 µM.

NOTE: At least a 0.7 mL final volume is needed per concentration tested.

1.3. ISCA filling

1.3.1. Fill a 1 mL syringe with the filtered chemoattractant and connect it to a 27 G syringe. Holding the ISCA with the port facing upward, slowly inject the substance until a small droplet appears on top of the port.

NOTE: 1) Each dilution or substance must be injected with a separate syringe and needle to prevent cross-contamination. 2) This small droplet is important because it ensures that no air bubble is trapped within the port, which could impair the ability of microbes to migrate through the port. 3) It is recommended to fill an entire row per substance or concentration (five wells) to provide an adequate minimal volume for further analyses. 4) One row per ISCA should act as negative control and should be filled with the filtered medium in which the microbes will be suspended. This treatment accounts for the effect of random motility by microbes into the ISCA wells and should be used to normalize the treatments containing a chemoattractant.

1.4. Deployment in the laboratory

1.4.1. Overnight, incubate a 5 mL culture enriched with 1% marine broth (for marine bacteria) or 1% lysogeny broth (LB, for fresh water bacteria).

NOTE: Motile bacterial isolates or natural bacterial communities can be used for laboratory deployments.

1.4.2. Incubate the culture for 12 h at room temperature (RT) and 180 rpm. After 12 h, ensure that the microbial communities are motile by direct observation under a microscope.

1.4.3. Spin down the culture at 1,500 x *g* for 10 min and resuspend 1/100 in 150 mL of appropriate medium (e.g., filtered seawater, filtered freshwater).

1.4.4. Place two small pieces of double-sided adhesive tape on the flat surface of a 200 mL capacity tray (the lids of 1 mL tip boxes contain the ideal dimensions for this purpose and can easily be autoclaved). Place one ISCA on top, ensuring that it attaches securely to the tape. Slowly fill the deployment tray with the bacterial solution using a 50 mL serological pipette.

NOTE: Fill the tray until the ISCA is submerged with approximately 1–2 cm of liquid. If using multiple trays, use the same volume across all.

1.4.5. Leave the ISCA incubate for 1 h to allow bacterial chemotaxis. After 1 h, remove the medium very gently with a 50 mL serological pipette to minimize turbulent flow.

1.4.6. Retrieve the ISCA from the deployment tray without touching the upper surface. Use a pipette and disposable wipers to remove remaining liquid on the ISCA surface.

NOTE: It is important to avoid touching the ports during this process, as the resulting changes in pressure can remove or add bacteria from the outside environment into the well and thereby bias the bacterial density and composition inside the well.

1.5. Retrieval of the samples

1.5.1. Holding the ISCA with the port facing downward, retrieve the volume of the wells using a sterile 27 G syringe needle attached to a 1 mL syringe.

NOTE: Each row (if containing the same substance) can be pooled to provide a working sample of approximately 550 µL. This sample can subsequently be aliquoted into different tubes depending on the required downstream applications.

1.5.2. Determine the number of bacteria attracted to each chemoattractant concentration by analyzing the samples with flow cytometry[12]. Choose the concentration of chemoattractant that maximizes chemotaxis for subsequent field deployments.

2. Preparation for field deployment

NOTE: Preparation of material and construction of the flow-damping enclosure (section 2) must be conducted prior to deployment.

2.1. Preparation of materials

2.1.1. Prepare all materials listed in **Table 1**.

NOTE: Material quantities are provided for one ISCA.

2.2. Construction and preparation of the flow-damping enclosure

NOTE: The flow-damping enclosure minimizes unwanted turbulences that otherwise prevents the establishment of chemical gradients emanating from the ISCA.

2.2.1. Cut the pieces for the deployment enclosure with a laser cutter from a 3 mm acrylic sheet.

NOTE: The file for the pieces can be found using the following link: <https://figshare.com/articles/Flow_damping_enclosure_for_ISCA_deployments/10630220>.

2.2.2. Assemble the laser-cut pieces as demonstrated in **Figure 2** using acrylic glue.

NOTE: Assemble the pieces with care. Holes or misalignment can create leaks upon deployment, which directly impacts data quality.

2.2.3. Leave the assembled enclosure to dry overnight.

2.2.4. Wash the enclosure with deionized water.

2.2.5. Identify potential leakage by pouring deionized water into the enclosure. Fix any potential leaking joints by adding more acrylic glue, then repeat steps 2.2.3–2.2.5.

2.2.6. Cut the screw threads into the acrylic piece that will be used to secure the ISCA. This can be achieved using a tap with a diameter and pitch matching the mounting screws.

2.2.6.1. First, affix the tap into a tap wrench, then secure the acrylic piece to be tapped in a benchtop vice. For the best results, make sure the acrylic piece is as level as possible. Make sure that the tap is perpendicular to the acrylic piece and start turning the tap wrench (clockwise), applying light pressure to the tap.

2.2.6.2. After several full revolutions in the acrylic piece, reverse the rotation of the tap (counterclockwise) for one-quarter of a rotation to clear acrylic from the tap. Repeat the process until the entire depth of the acrylic piece is tapped.

2.2.6.3. Finally, remove the tap (turning counterclockwise) and test the threads using a screw.

3. Procedure in the field

3.1. Water filtration

3.1.1. Collect water from the field site when ready to start the experiment. Filter 5 mL of water per ISCA through a 0.2 µm syringe filter (with a 50 mL syringe) into a 50 mL conical centrifuge tube.

NOTE: Approximately 3 mL of filtered water are required to fill all the wells of an ISCA; however, it is recommended to 1) filter 5 mL per device to account for losses during the quadruple filtration process, and 2) preserve aliquots of the filtrates as negative controls for both flow cytometry and molecular procedures.

3.1.2. Filter the filtrate 2x through a 0.2 µm hydrophilic GP filter cartridge (using the same one, both times) with a new 50 mL syringe into a new 50 mL conical centrifuge tube. Filter the filtrate through a 0.02 µm syringe filter (with a new 50 mL syringe) into a new 50 mL conical tube.

NOTE: This quadruple filtration should remove nearly all microorganisms and particles from the water. Keep the final filtrate away from any source of heat until use. This water will be used to resuspend all chemicals used in the ISCA, and it should be maintained at the same temperature as the water at the deployment site. Convective flows triggered by differences in temperature between the ISCA wells and outside environment may otherwise occur.

3.1.3. Use aliquots of the filtrate to resuspend all chemoattractants of interest (typically dry) to the desired concentrations in 15 mL conical centrifuge tubes.

3.1.4. Filter the resuspended chemoattractants through a 0.2 µm syringe filter with a 10 mL

syringe into sterile 15 mL conical centrifuge tubes to remove unwanted particles or water-insoluble compounds (if using extracts).

NOTE: Filter gently to prevent particles from passing through the filter. It is important to resuspend the chemoattractants in the ultrafiltered water from the field site and not solubilize them into other solutions. Using water from the field site is necessary to (1) obtain the same salt concentration inside the wells as that in the bulk environmental water to prevent density-driven flow, and (2) guarantee that background nutrient levels are equal inside and outside of the well.

3.2. ISCA filling

3.2.1. Perform section 1.3 to fill the ISCA.

NOTE: It is recommended to fill one row (five wells) per substance (i.e., three different substances per ISCA and one ultrafiltered seawater control).

3.3. Deployment in the field

3.3.1. Screw the ISCA (**Figure 3A**) to piece 9 of the enclosure (**Figure 2K and 3B**).

NOTE: The flow-damping enclosure outlined above can contain two ISCAs side-by-side or one ISCA placed at its center.

3.3.2. Close the enclosure (**Figure 3C**) and seal it with adhesive tape (**Figure 3D**).

NOTE: Wrinkles must be avoided to ensure a perfect seal. Seal all sides first, then (in a second step) seal the side holes, which will be used to drain water from the enclosure at the end of sampling. Do not seal the top and bottom holes. Do not place the ISCA upside down, as density-driven flow can occur in wells containing chemoattractants, which will bias the number of cells in the wells.

3.3.3. Because the enclosure must remain steady during deployment, it is recommended to attach it to manmade structures (e.g., pontoon, ladder) using bungee cords.

NOTE: The enclosure can be attached to a deployment arm (here, a modified clamp with a perpendicular platform) using bungee cords before immersion in the water. Alternatively, the enclosure can be filled and secured with a small weight on shallow substrates. If deployments are intended in the pelagic ocean, the enclosure can be attached to a net with a buoy on one side and dive weight on the other.

3.3.4. Submerge the enclosure completely to start filling. While filling, hold the enclosure firmly to prevent excessive water movement inside. Once the level of the water reaches the top of the enclosure, make sure that no air is trapped inside.

NOTE: In case some air bubbles are trapped, tilt the enclosure gently with the vent hole facing upward, which will enable the bubbles to escape.

3.3.5. Once completely full, seal the bottom and top holes with two plugs, which can be made out of silicon or rubber or by sealing the extremities of 20 µL pipette tips (**Figure 4**).

NOTE: This step prevents flow inside the enclosure during sampling.

3.3.6. Leave the ISCA in place for sampling for 1–3 h.

NOTE: The ideal deployment time is primarily dictated by the temperature of the water and doubling time of the bacterial community. When the water temperature is above 20 °C, it is not recommended to deploy the ISCA for more than 1 h, because cell division can occur in the wells containing chemoattractants after 1.5–2.0 h. However, optimal deployment time can be tested prior to the ISCA experiment by amending natural communities with the loaded chemicals and quantifying the number of cells through time.

3.3.7. Remove the enclosure from the water. Place it over a container enabling the water to be drained from the enclosure.

3.3.8. Remove the upper part of the adhesive tape from the front holes very gently.

NOTE: The flow of the water leaving the enclosure must be at a dripping speed. Proceed one hole at a time, from the top of the enclosure to the bottom. It should take approximately 10–15 min to drain the enclosure completely.

3.3.9. Once the waterline passes below the top of the ISCA, remove the bottom plug, and drain the rest of the water.

3.3.10. While the ISCAs are still attached to the enclosure, carefully remove the water trapped on top of the ISCA with a 1 mL pipette.

3.3.11. Remove the ISCA without touching the upper surface and use a disposable wipe to remove any remaining liquid on the surface.

NOTE: It is important not to touch the ports during this process, as the resulting changes in pressure can remove or add bulk bacteria into the well and bias the bacterial counts.

3.3.12. Retrieve the samples from the ISCA by repeating step 1.5.1.

4. **Downstream applications**

NOTE: Volumes are given based on a 550 µL sample (one row of an ISCA).

4.1. Fix 100 µL of well contents with glutaraldehyde (2% final concentration) for flow cytometry to quantify chemotaxis to each attractant.

NOTE: Store on ice (or at 4 °C) and analyze the samples on the same day. Alternatively, samples can be flash frozen in liquid nitrogen following fixation if analysis is not feasible on the same day. Flow cytometry is the recommended method to quantify the number of cells in the ISCA wells, as it is straightforward, fast, and accurate[12].

4.2. Snap freeze 300 µL of well content in liquid nitrogen for subsequent DNA extraction and analysis[11].

NOTE: Store the samples at -80 °C until analysis.

4.3. Add 90 µL of well contents to 10 µL of TE-glycerol buffer and snap-freeze the samples for single-cell genomics[13].

4.4. Spread 10–20 µL on agar plates containing the desired medium for bacterial isolation.

REPRESENTATIVE RESULTS:

This section presents laboratory results using the ISCA to test the chemotactic response of marine microbes to a concentration range of glutamine, an amino acid known to attract soil bacteria[14]. The concentration of glutamine that elicited the strongest chemotactic response in the laboratory tests was used to perform a chemotaxis assay in the marine environment.

To perform the laboratory tests, seawater communities sampled from coastal water in Sydney, Australia, were enriched for motile cells through a simple nutrient amendment[4], as described in step 1.4. Glutamine was serially diluted in ultrafiltered seawater to obtain final concentrations ranging from 10 mM to 100 µM. Five ISCA replicates were deployed simultaneously for this experiment, and each contained three different glutamine concentrations (one concentration per row) as well as a filtered seawater control row. After a 1 h deployment, the contents of each ISCA row (containing the same glutamine concentration) were pooled to provide working samples of approximately 550 µL. This volume was fixed in glutaraldehyde (2% final concentration), and the number of responding bacteria quantified via flow cytometry.

Briefly, bacterial abundance was quantified by 1) staining the cells with a green fluorescent DNA dye and 2) analysis using a flow cytometer with ultrafiltered deionised water as the sheath fluid. For each sample, forward scatter (FSC), side scatter (SSC), and green fluorescence were recorded. The samples were analysed at a flow rate of 25 µL/min, with bacterial cells discriminated according to SSC and green fluorescence. The chemotactic index (I_c) was determined by dividing the bacterial counts present in each sample by the averaged bacterial counts in the filtered seawater control wells (FSW).

Results showed that 1 mM was the optimal glutamine deployment concentration, as it induced

a significant chemotactic response that was 18-fold higher than the filtered seawater control (*t*-test, $p < 0.001$) (**Figure 5A**). Higher or lower concentrations of glutamine induced significant but weaker chemotactic responses (I_c = 5.43 for 100 µM, $p < 0.001$; I_c = 7.34 for 10 µM, $p < 0.001$). If the chemoattractant concentration added to an ISCA well is too high, chemotaxis into the well can be reduced, because (1) bacteria will not be able to detect a gradient in the port section and may aggregate above the well, or (2) the pH or osmolarity of the well may be affected.

The optimal glutamine concentration was subsequently used for field deployment. Five ISCA replicates filled with 1 mM glutamine were deployed for 1 h at a coastal site near Sydney, Australia (33.91 °S, 151.26 °E). Glutamine attracted 2.98x more bacteria than the control wells filled with filtered seawater from the deployment site (**Figure 5B**). The chemotactic response in this field experiment was significantly different from the controls (*t*-test, $p < 0.001$) and constituted a strong response for coastal seawater[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Detailed views of the in situ chemotaxis assay (ISCA). (A) The latest injection-molded ISCA. (**B**) Schematic of an ISCA well. Scale bar = 7.463 mm.

Figure 2: Assembly of the flow-damping enclosure. (A) The pieces required for assembly of the deployment enclosure. During fabrication, edges should be smooth. (**B**) Place pieces 2a, 2b, 3a, and 3b around piece 1 (lower surface). (**C**) Assemble the lower part of the enclosure by putting a thin layer of acrylic glue around the first edge of the lower surface (1). **(D)** Place the first longer sidewall piece (2a) vertically onto the glue and hold it in place. The glue requires ~1 min to solidify and allows piece 2a to support itself while placing the next element. (**E)** Apply a thin layer of acrylic glue to the lower surface on a shorter side of piece 1. Place the short sidewall piece (3a) onto the acrylic glue and lock it into the previously placed sidewall. Hold the two pieces for approximately 1 min. **(F)** Place the other short sidewall piece (3b) onto the opposite side of the lower surface (1). Again, lock it into the connecting piece (2a) and hold it for approximately 1 min. **(G)** If needed, reapply acrylic glue to the remaining long side of the lower surface (1). Place the last long sidewall piece (2b) and connect it into the two adjacent pieces (3a and 3b). Make sure that all the pieces are properly aligned with the lower piece (1) and that no signs of misalignment or gaps between sidewalls or the lower surface are present. Repeat these steps to assemble the complementary upper portion of the enclosure (4, 5a, 5b, 6a, and 6b). (**H)** Make sure that the hole in the corner of piece 4 is not obstructed during assembly, otherwise punch the opening with a sharp object such as a needle. The holes of the enclosure play a critical role in the deployment process and allow water to drain in a slow and controlled manner. Their diameter has been optimized to reduce turbulent flow inside the enclosure, which prevents disturbance of the fluid surrounding ISCA ports upon retrieval. (**Ia,b)** Glue together two large rectangles (7) and separately glue two smaller ones (8). Repeat once for each. (**J)** Glue the four assembled rectangles in the center of the enclosure's lower surface (1). (**K)** Glue the upper deck (9) on top of the rectangles (7 and 8). Make sure that the side holes of the piece are on the external side of the rectangles.

Figure 3: **Placement of ISCAs in the enclosure and sealing by taping**. (**A**) Place the mounting screws in the ISCA. (**B**) ISCA placement in the deployment enclosure. Place the ISCA in the middle of the deployment enclosure and attach it with the specified screws. The lower drain hole of the enclosure must be sealed with a modified 20 µL tip (**Figure 4**) once the enclosure is filled with water. This helps to avoid generation of turbulent flows that can affect the stability of the chemical field and effectiveness of chemotaxis. (**C**) The upper and lower parts are assembled together. (**D**) Sealing of the enclosure using adhesive tape. Wrinkles must be avoided to prevent leaks.

Figure 4: Plug for sealing the flow-damping enclosure. The plug can be made by sealing a 20 µL pipette tip with heat.

Figure 5: Chemotaxis assays using the ISCA toward glutamine of an enriched motile community in the laboratory and natural microbial population in the field. Chemotaxis index I_c (representing the concentration of cells within ISCA wells) normalized by the mean concentration of cells within the wells containing the filtered seawater (FSW) after 60 min of deployment. Each concentration was tested in five ISCA replicates. Bacterial cells were quantified by flow cytometry (A): FSW = 4.46 ± 0.25 x 10^3; 100 µM = 2.43 ± 0.16 x 10^4; 1 mM = 8.07 ± 0.45 x 10^4; 10 mM = 3.28 ± 0.20 x 10^4; (B): FSW = 1.26 ± 0.11 x 10^4; 1 mM = 3.76 ± 0.28 x 10^4 cells/mL). All concentrations of glutamine tested in the **(A)** laboratory and **(B)** field induced a chemotactic response significantly higher than the filtered seawater (FSW) controls**.** In all pairwise comparisons: (A) Tukey HSD, $n = 5$, $p < 0.005$; (B) Tukey HSD, $n = 5$, $p < 0.005$. Error bars represent SEM.

Table 1: **Materials necessary for field deployment.**

DISCUSSION:

At the scale of aquatic microorganisms, the environment is far from homogenous and is often characterized by physical/chemical gradients that structure microbial communities[1,15]. The capacity of motile microorganisms to use behavior (i.e., chemotaxis) facilitates foraging within these heterogeneous microenvironments[1]. Studying chemotaxis directly in the environment has the potential to identify important interspecific interactions and chemical preferences, and this can help untangle the contributions of specific microbes to biogeochemical processes. The presented protocol deploys the ISCA in the environment[11] to facilitate the acquisition of reproducible research on chemotaxis in situ.

Using the ISCA, it is shown that glutamine elicits a positive chemotactic response both in laboratory conditions and in the field. The ISCA deployment of glutamine in the field yields a lower chemotactic response than in the laboratory (**Figure 5**). Similar patters between laboratory and field experiments have been observed previously[11]. These can be explained by the lower proportion of motile cells in the environment compared to the enriched communities or single motile isolate used in laboratory assays.

The importance of preliminary laboratory-based experiments should not be underestimated, as

they permit determination of optimal chemoattractant concentrations to use in field deployments. The optimal concentration is specific to each chemoattractant and influenced by its molecular weight, solubility, and diffusivity from the wells. In the case of deployment of multiple distinct substances, each should be tested individually across a concentration range. If no chemotaxis is detected in the field after 1 h, longer deployments can be carried out. However, the length of the deployment is strongly constrained by bacterial growth and should always be shorter than the division rate of bacteria in the targeted environment. This helps to avoid population growth within the ISCA.

The ISCA is sensitive to water turbulence and care should be taken when filling and emptying the flow-damping enclosure. These steps must be performed slowly, because flows resulting from rapid filling can flush or dilute the contents of the wells. As a result, this removes or prevents chemoattractants from diffusing properly or introducing bacteria from the surrounding environment, ultimately biasing cell counts. Fully filling the enclosure with water while venting all air, then closing it completely, ensures that turbulence will not interfere with the deployment. Collecting metadata at the deployment site (i.e., temperature, salinity, chlorophyll/nutrient concentration) is also a critical step to interpret results, as these factors can influence chemotaxis.

The ISCA is an accessible, user-friendly device that provides new insights into the role and prevalence of chemotaxis in the environment. It enables interrogation of chemotaxis in any system containing a liquid phase (e.g., marine, freshwater, soil, wastewater systems). Finally, it can be used for targeted studies on pathogens and antibiotic resistance in the environment, isolation of key microbes for bioprospecting, and bioremediation of specific pollutants and microplastics.

ACKNOWLEDGMENTS:

This research was funded in part by the Gordon and Betty Moore Foundation Marine Microbiology Initiative, through grant GBMF3801 to J.R.S. and R.S., and an Investigator Award (GBMF3783) to R.S., as well as an Australian Research Council Fellowship (DE160100636) to J.-B.R., an award from the Simons Foundation to B.S.L. (594111), and a grant from the Simons Foundation (542395) to R.S. as part of the Principles of Microbial Ecosystems (PriME) Collaborative.

DISCLOSURES:

The authors declare no conflict of interest.

REFERENCES:

1. Stocker, R. Marine microbes see a sea of gradients. *Science.* **338**, 628–633 (2012).
2. Raina, J.-B., Fernandez, V., Lambert, B., Stocker, R., Seymour, J. R. The role of microbial motility and chemotaxis in symbiosis. *Nature Reviews Microbiology.* **17**, 284–294 (2019).
3. Chet, I., Asketh, P., Mitchell, R. Repulsion of bacteria from marine surfaces. *Applied Microbiology.* **30**, 1043–1045 (1975).
4. Smriga, S., Fernandez, V. I., Mitchell, J. G., Stocker, R. Chemotaxis toward phytoplankton drives

organic matter partitioning among marine bacteria. *PNAS.* **113**, 1576–1581 (2016).
5. Matilla, M., Krell, T. The effect of bacterial chemotaxis on host infection and pathogenicity. *FEMS Microbiology Reviews.* **42**, (2018).
6. Adler, J. Chemotaxis in bacteria. *Science.* **153**, 708–716 (1966).
7. Adler, J., Dahl, M. M. A method for measuring the motility of bacteria and for comparing random and non-random motility. *Journal of General Microbiology.* **46**, 161–173 (1967).
8. Ahmed, T., S. Shimizu, T., Stocker, R. Microfluidics for bacterial chemotaxis. *Integrative Biology* **2**, 604–629 (2010).
9. Hol, F. J. H., Dekker, C. Zooming in to see the bigger picture: microfluidic and nanofabrication tools to study bacteria. *Science.* **346**, 1251821 (2014).
10. Rusconi, R., Garren, M., Stocker, R. Microfluidics expanding the frontiers of microbial ecology. *Annual Review of Biophysics.* **43**, 65–91 (2014).
11. Lambert, B. S. *et al.* A microfluidics-based in situ chemotaxis assay to study the behaviour of aquatic microbial communities. *Nature Microbiology.* **2**, 1344–1349 (2017).
12. Marie, D., Partensky, F., Jacquet, S., Vaulot, D. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Applied Environmental Microbiology.* **63**, 186–193 (1997).
13. Rinke, C. *et al.* Obtaining genomes from uncultivated environmental microorganisms using FACS–based single-cell genomics. *Nature Protocols.* **9**, 1038–1048 (2014).
14. Gaworzewska, E. T., Carlile, M. J. Positive chemotaxis of Rhizobium leguminosarum and other bacteria towards root exudates from legumes and other plants. doi:10.1099/00221287-128-6-1179 (1982).
15. Walker, T. S., Bais, H. P., Grotewold, E., Vivanco, J. M. Root exudation and rhizosphere biology. *Plant Physiology.* **132**, 44–51 (2003).

Figure 1

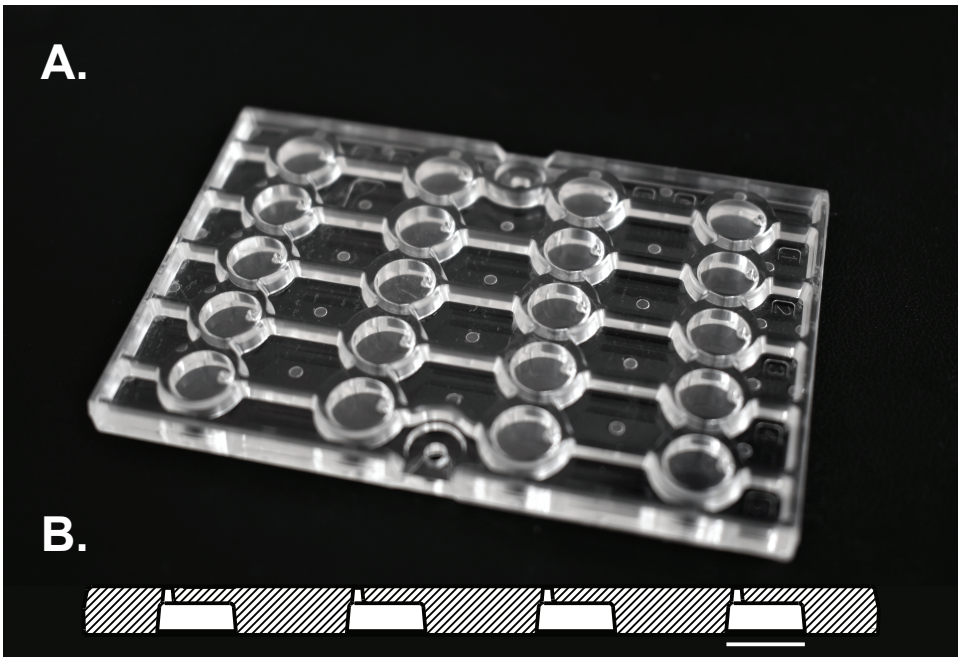


Figure 2

Click here to access/download;Figure;Figure 2_final.pdf

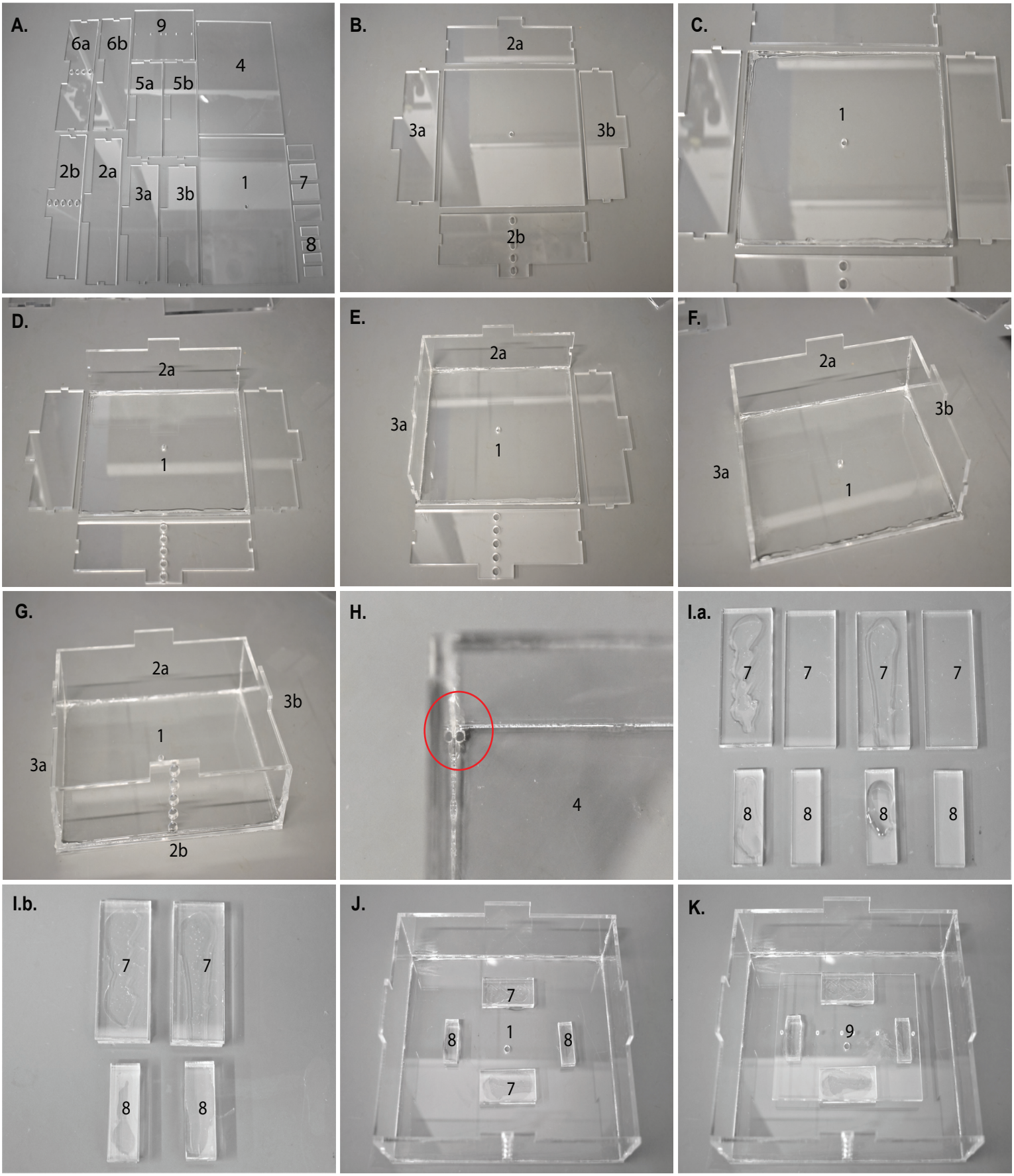


Figure 3

Click here to access/download;Figure;Figure 3_final.pdf

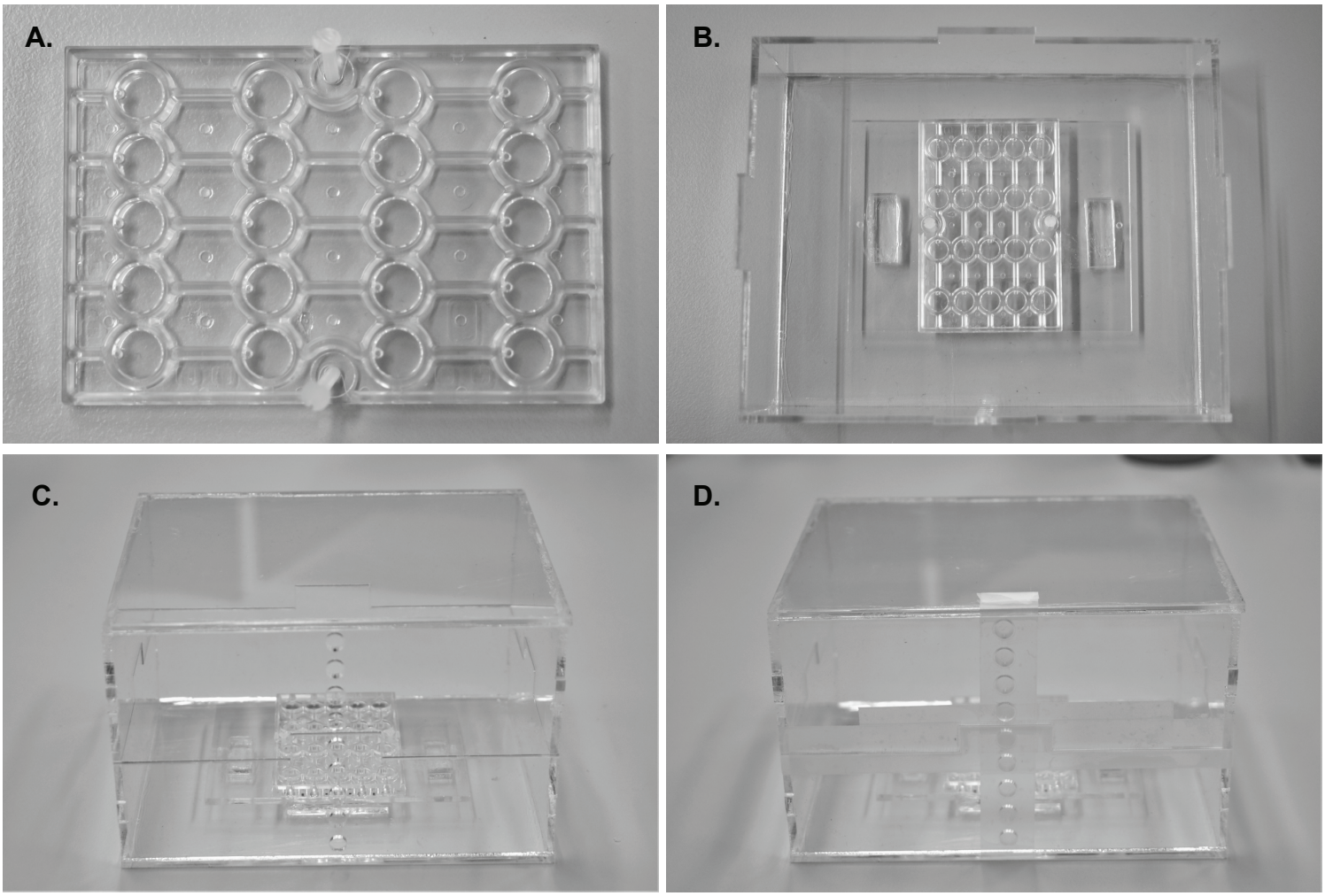


Figure 4

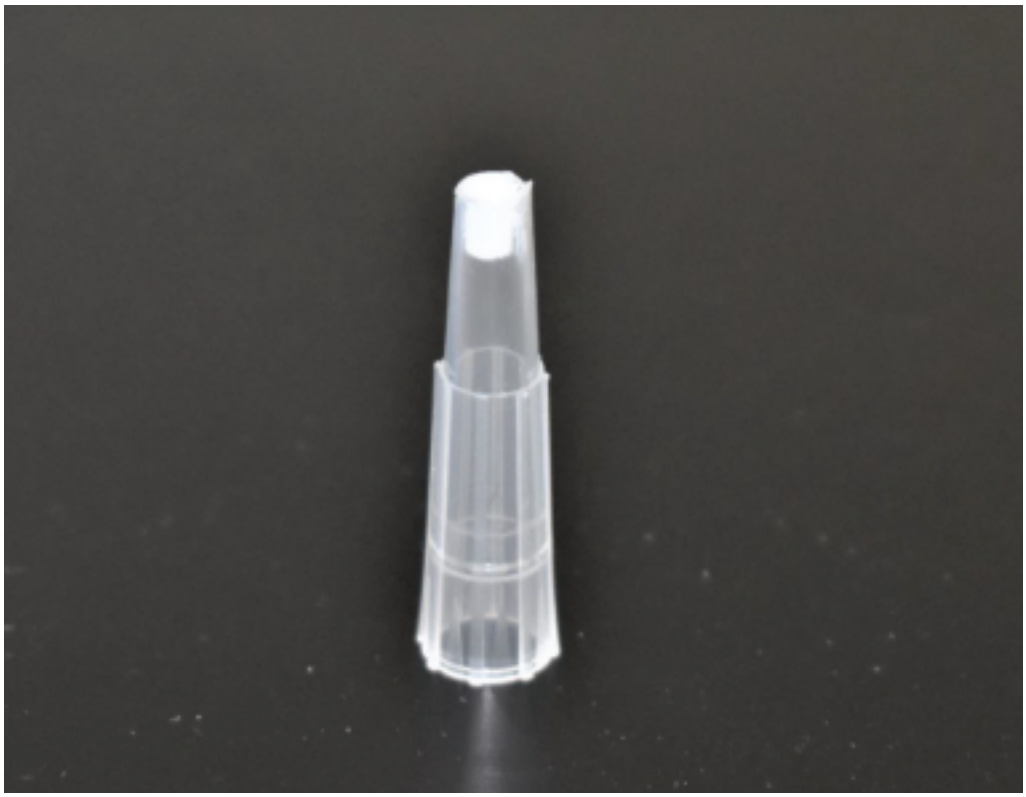
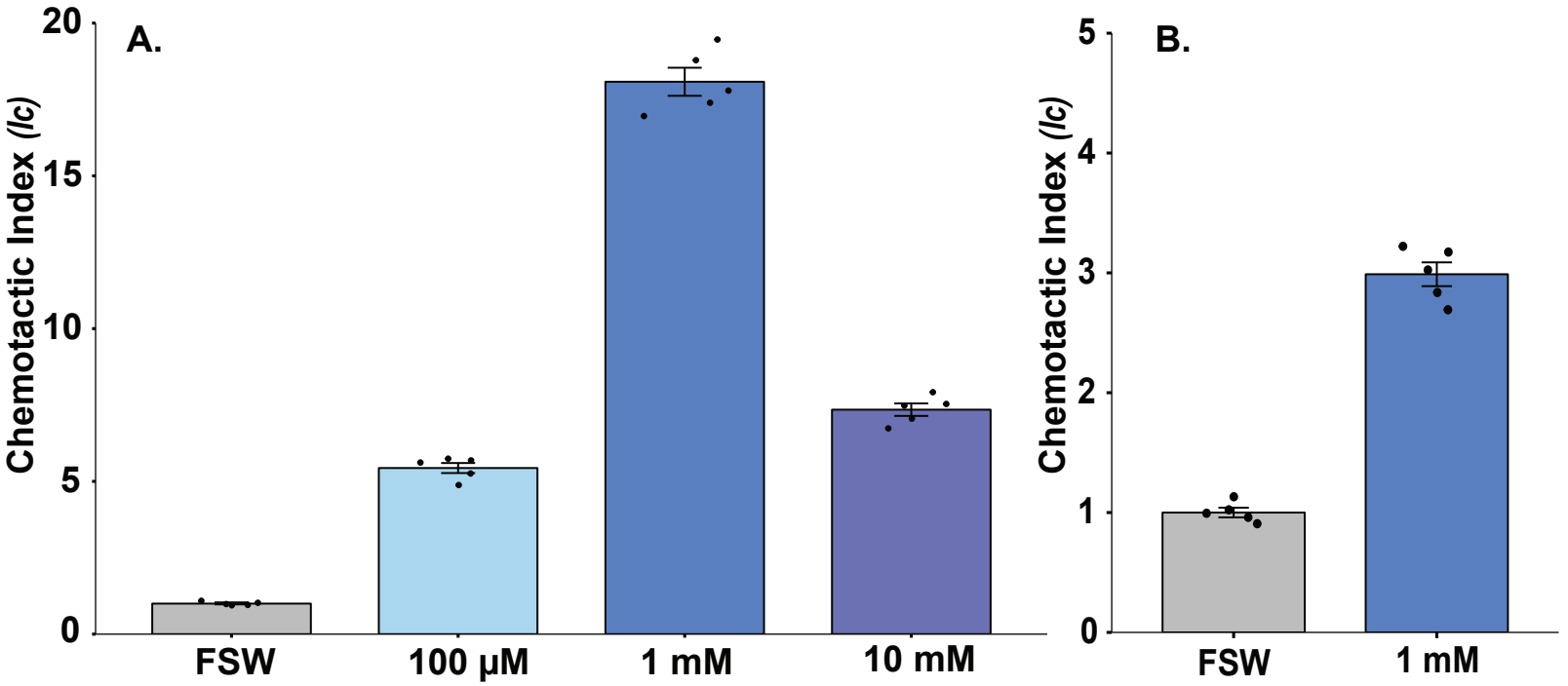


Figure 5

Click here to access/download;Figure;Figure 5.pdf



Field material	Quantity
Water filtration	
Tube rack - 50 mL	1
Hydrophilic GP filter cartridge - 0.2 µm	1
Syringe filter - 0.2 µm	1
Syringe filter - 0.02 µm	1
Syringes - 50 mL	4
Conical centrifuge tube - 50 mL	5
Chemical resuspension	
Conical centrifuge tube - 15 mL	4
Chemoattractants	
Syringes - 10 mL	4
Syringe filter - 0.2 µm	4
Tube rack - 15 mL	1
ISCA filling	
Syringe needle 27G	4
Syringe - 1 mL	4
ISCA	1
Deployment	
Deployment enclosure	1
Nylon slotted flat head screws	2
Deployment arm	1
Bungee cord	2
Adhesive tape	1
Deployment enclosure plug	1
Samples retrieval	
Syringe needle 27G	4
Syringes - 1 mL	4
Centrifuge tube - 2 mL	12
Disposable wipers	1 box
Glutaraldehyde 25%	10 mL
Other material	
Pipettes set	1
Pipette tips 200 µL	1 box
Pipette tips 20 µL	1 box
Pipette tips 1 mL	1 box

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
Acrylic glue	Evonik	1133
Acrylic sheet	McMaster-Carr	8505K725
Adhesive tape	Scotch	3M 810
Autoclave	Systec	D-200
Benchtop centrifuge	Fisher Scientific	75002451
Bungee cord	Paracord Planet	667569184000
Centrifuge tube - 2 mL	Sigma Aldrich	BR780546-500EA
Conical centrifuge tube - 15 mL	Fisher Scientific	11507411
Conical centrifuge tube - 50 mL	Fisher Scientific	10788561
Deployment arm	Irwin	1964719
Deployment enclosure plug	Fisher Scientific	21-236-4
Disposable wipers	Kimtech - Fisher Scientific	06-666
Flow cytometer	Beckman	C09756
Glutaraldehyde 25%	Sigma Aldrich	G5882
Green fluorescent dye	Sigma Aldrich	S9430
Hydrophilic GP filter cartridge - 0.2 µm	Merck	C3235
In Situ Chemotaxis Assay (ISCA)	-	-
Laser cutter	Epilog Laser	Fusion pro 32
Luria Bertani Broth	Sigma Aldrich	L3022
Marine Broth 2216	VWR	90004-006
Nylon slotted flat head screws	McMaster-Carr	92929A243
Pipette set	Fisher Scientific	05-403-151
Pipette tips - 1 mL	Fisher Scientific	21-236-2A
Pipette tips - 20 µL	Fisher Scientific	21-236-4
Pipette tips - 200 µL	Fisher Scientific	21-236-1
Sea salt	Sigma Aldrich	S9883
Serological pipette - 50 mL	Sigma Aldrich	SIAL1490-100EA
Syringe filter - 0.02 µm	Whatman	WHA68091002
Syringe filter - 0.2 µm	Fisher Scientific	10695211
Syringe needle 27G	Henke Sass Wolf	4710004020
Syringes - 1 mL	Codau	329650
Syringes - 10 mL	BD	303134
Syringes - 50 mL	BD	15899152
Tube rack - 15 mL	Thomas Scientific	1159V80
Tube rack - 50 mL	Thomas Scientific	1159V80
Uncoated High-Speed Steel General Purpose Tap	McMaster-Carr	8305A77
Vacuum filter - 0.2 µm	Merck	SCGPS05RE

Comments
Acrifix 1S 0116
Or different company
Scotch Magic tape
Or different company
Or different company
Or different company
Eppendorf tube
Falcon tube
Falcon tube
Or different company
See alternatives in manuscript
Kimwipes
CYTOFlex
Or different company
SYBR Green I - 1:10,000 final dilution
Sterivex filter
Contact corresponding authors
Or different company
Or different company
Difco
M 2 × 4 × 8 mm
Or different company
Or different company
Or different company
Or different company
For artificial seawater
Or different company
Anatop filter
Or different company
0.4 × 12 mm
Insulin Luer U-100
Or different company
Or different company
Or different company
Or different company
Or different company
Steritop filter

Zürich, 30.01.2020

Dear Dr. Phillip Steindel,

We would like to thank you for your time in handling our manuscript, "Deployment of the *In Situ* Chemotaxis Assay (ISCA) to examine microbial behavior in aquatic ecosystems" by Estelle E. Clerc, Jean-Baptiste Raina, Bennett S. Lambert, Justin Seymour & Roman Stocker.
We would like to also thank the Reviewers for their constructive comments and excellent suggestions.

For ease of editorial review, we have included the Editor and Reviewers comments in **bold**, with our responses in *italics*. Text that has been *inserted or changed is underlined.*

Best regards,
Estelle E. Clerc (on behalf of all authors)

Response to Editor

General

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been thoroughly proofread and any spelling or grammar issues have been corrected.

2. Please provide at least 6 key words or phrases.

We have added Line 28: "Behavioral assay" as last key word.

3. Please clarify-is this intended to be filmed both in the field and in the lab?

Our protocol is intended to be filmed both in the lab and in the field. The laboratory component will cover all the preparation steps (parts 1 and 2 in the manuscript), while the field component will cover the deployment of the device in situ (part 3 in the manuscript). Please let us know if filming in both environments will present additional challenges for the crew.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Steritop, Sartorious, Kimwipe, Falcon, Eppendorf, Sterivex, Anatop

We have now removed all commercial language throughout the manuscript.

Protocol

1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have carefully proofread the protocol and have made sure to address how each step is performed. Additional references have been added for further clarification

Specific Protocol steps

1. 1: Please provide information about the creation of the ISCA (or information on how to otherwise obtain it).

We thank the Editor for this comment. We have now added the following text, Lines 83-84, to address this point: "For interest in the latest version of the device, the corresponding authors can be contacted".

2. 3.3.4 NOTE: Is there supposed to be an attached video, or do you mean the video to be filmed by us?

We thank the Editor for his question. To illustrate the deployment procedure, we were planning to use the field sequence filmed by JoVE.

Figures

1. Figure 1: Please provide this figure as 1 page or split into 2 separate figures (i.e., Figures 1 and 2).

Figure 1 has been modified and is now a single-page figure. Note that Figure 1 has been renamed as Figure 2 after modification of the manuscript.

2. Figure 1I-K: Are pieces 7 and 8 triangles or rectangles?

*We apologize for this oversight. We have edited the text, Lines 483-486, to correct this error: "**I.a and I.b.** Glue together two large rectangles (7) and separately glue two smaller ones (8). Repeat once for each. **J.** Glue the four assembled rectangles in the center of the enclosure's lower surface (1)."*

3. Figure 2D: Please provide a legend for this panel.

*We thank the Editor for this comment. We have altered the text Lines 494-496: "**C.** The upper and lower parts are assembled together. **D.** Sealing of the enclosure using adhesive tape. Wrinkles must be avoided to prevent leaks."*

Table of Materials

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have fully proofread the Table of Materials and ensured that all materials and equipment used were mentioned.

Response to Reviewer #1

This manuscript has presented an idea to design a support structure for a previously published microfluidic device to monitor chemotaxis in natural environment. They have presented the step-by-step arrangement of the device that supports/ encloses a microfluidic device inside so that the microfluidic device can be deployed in the natural environment directly which is relatively difficult

to achieve in microfluidic devices alone.

Overall, this manuscript has the potential to achieve the desired result. However, in my opinion, I recommend this study to go through some minor corrections that are listed below.

We thank the Reviewer for this positive feedback.

Minor Concerns

1. As explained in a note (Lines 258-262), the composition of the solution to which chemoattractant should be dissolved need to be same, thus it is not clear why it is necessary to filter the water (explained in section 3.1.3-3.1.7) when it is finally going to be mixed with unfiltered water. Other than that, even if the chemoattractant solution in filtered water is mix with the unfiltered water, the concentration of the salt will be different as the bulk environmental water (Lines 260-261).

Sections 3.1.2-3.1.7 describe how the water from the deployment site needs to be sequentially filtered to remove most of the microorganisms. This water is then used to suspend the chemoattractants, which are typically in powder form. There is no subsequent mixing with unfiltered seawater. To address this confusion, we have now modified the text Lines 292-293: "Use aliquots of the filtrate to resuspend all of the chemoattractants of interest (typically dry) to the desired concentrations in 15 ml conical centrifuge tubes."

2. Describe the importance of holes in the device more precisely.

We are not sure if the Reviewer is referring to the port of the ISCA or the holes of the enclosure.

(1) The ports of the ISCA constitute the link between the wells containing the putative chemoattractants and the outside environment. Each well has only one port, used to fill the wells prior deployment, and from which the putative chemoattractant diffuse out creating concentration gradients. Motile and chemotactic bacteria can then respond to these gradients by swimming inside the wells through the port. Much of this description is already in the manuscript Lines 84-88. In addition, we have now added a new Figure (Figure 1) clearly showing how the device looks and the ports.

(2) The holes of the enclosure are essential to drain the water inside the box very slowly. They minimize the flow velocity inside the box and ensure that the well contents are not disturbed while

the ISCA are being recovered from the field. To address this point, the following sentence has been added to the manuscript Lines 480-483: "The holes of the enclosure play a critical role in the deployment process and allow water to drain in a slow and controlled manner. Their diameter has been optimized to reduce turbulent flow inside the enclosure, which prevents disturbance of the fluid surrounding ISCA ports upon retrieval."

3. In the figure legend (Figure 1), there is no triangle shown in the image. (This part is however present in the previously published article "A microfluidics-based in situ chemotaxis assay to study the behavior of aquatic microbial communities") although there is a mention of triangles (some part of the device).

*We apologize for this oversight. The flow-damping enclosure has been slightly modified from the original publication to more securely attach the new injection molded ISCA and the mention of triangles was a mistake. We have edited the text at Lines 483-486: "**I.a and I.b.** Glue together two large rectangles (7) and separately glue two smaller ones (8). Repeat once for each. **J.** Glue the four assembled rectangles in the center of the enclosure's lower surface (1)."*

Response to Reviewer #2

In general, this manuscript is well-written and the unique feature of this protocol for in-field chemotaxis test is very interesting and can be useful for the relevant research communities. A few comments/questions for the authors to consider:

We thank the Reviewer for this positive assessment.

1. The description of the device is not very clear in this protocol. It will be helpful to include more details and illustrations to aid readers' understanding.

*We thank the Reviewer for this comment. We have now added a new Figure (now Figure 1), showing the ISCA and its features in greater details. We have also edited the text Lines 84-86: "The ISCA is credit card-sized and consists of 20 wells distributed in a 5 by 4 array, each linked to the external aquatic environment by a small port (800 µm diameter) **(Figure 1)**."*

2. Do I understand it correctly that for the in-field test, cell count after the assay still requires specialized lab facilities such as flow cytometers? Even if this is the case, this protocol is still useful. But do the authors envision further development of the protocol so cell quantification can be also done in-field or only requires some simple instrument? This may be at least useful to discuss in the paper.

The Reviewer is correct, post-processing of the samples requires to count cells via flow cytometry. We have chosen this technique because it provides reliable, fast and precise quantification of cell numbers, while only requiring a small volume of sample (100 µl). Although we have considered other quantification techniques, such as DAPI counts, none of the alternatives were as robust and rapid as the use of flow cytometry. Samples can be flash frozen in liquid nitrogen following fixation if no instrument is available. We have now added a sentence to the manuscript to cover these points Lines 406-407:" NOTE 3: Flow cytometry is the recommended method to quantify the number of cells in the ISCA wells, as it is straightforward, fast and accurate (Marie et al., 1997)."

Response to Reviewer #3

The authors provide a detailed protocol for deploying their in situ chemotaxis assay (ISCA) to collect data from aquatic environments.

Minor Concerns
General comments

1. L268: In addition to testing different concentrations of chemoeffectors in the laboratory to find the optimal concentration, I would recommend also testing a range of concentrations within the device when it is deployed as it's always difficult to anticipate what microorganisms are present and what unknown environmental factors could affect the concentration of the chemoeffector.

We agree with the Reviewer's comment, testing a range of chemoattractant concentrations in the field is indeed highly recommended before running an extensive experiment. We have added the following sentence Lines 116-117: "Ideally, a concentration range should also be tested in the field in order to confirm laboratory results."

2. Show more detail of the port to the sample chamber.

*We thank the Reviewer for this comment. We have now added a new Figure showing the new ISCA and its wells in greater details. We have also edited the text Lines 84-86: "The ISCA is credit card-sized and consists of 20 wells distributed in a 5 by 4 array, each linked to the external aquatic environment by a small port (800 µm diameter) (**Figure 1**)."*

Specific comments

1. Is the injection molded ISCA device available for purchase? How would one obtain that part?

We thank the Reviewer for this comment. We have now added the following text, Lines 83-84, to address this point: "For interest in the latest version of the device, the corresponding authors can be contacted".

2. L190 Are there alternative approaches to flow cytometry that can be used?

We have chosen flow cytometry because this technique provides reliable, fast and precise quantification of cell numbers, while only requiring a small volume of sample (100 µl). Although we have considered other quantification techniques in early development phases, such as DAPI counts, none of the alternatives were as robust and rapid as flow cytometry. Samples can be flash frozen in liquid nitrogen following fixation if no instrument is available. We have now added a sentence to the manuscript to cover these points Lines 406-407:" NOTE 3: Flow cytometry is the recommended method to quantify the number of cells in the ISCA wells, as it is straightforward, fast and accurate (Marie et al., 1997)."

3. L210-211: It's not clear to me what is meant by the phrase "Tap the piece that will hold the ISCAs screwed in place." Provide more detail here.

We thank the Reviewer for this comment. Here we mean: making screw threads in a hole, where a screw will be inserted. This sentence has been modified Lines 250-259 and now reads: "2.2.7 Cut the screw threads into the acrylic piece that will be used to secure the ISCA. This is achieved using a tap with diameter and pitch matching the mounting screws. First, affix the tap into a tap wrench, then secure the acrylic piece to be tapped in a benchtop vice. For the best result make sure the acrylic piece is as level as possible. Make sure that the tap is perpendicular to the acrylic piece and start turning the tap wrench (clockwise), applying light pressure to the tap. After a couple of full revolutions in the acrylic piece,

reverse the rotation of the tap (counterclockwise) for a quarter rotation to clear acrylic from the tap. Repeat the process until the entire depth of the acrylic piece is tapped. Finally, remove the tap (turning counterclockwise) and test the threads using a screw."

4. L268: Instead of four different substances, I would recommend three and a buffer control to be consistent with previous instruction to incorporate a control (L144-147).

We agree with the Reviewer and edited the sentence Lines 313-314 to clarify this point: "NOTE: We recommend filling one row (5 wells) per substance (i.e., three different substances per ISCA and one ultrafiltered seawater control). "

5. L273: Give more details about what part of the enclosure the ISCA is being screwed into.

*We thank the Reviewer for asking for clarification. We have edited the text Line 318: "3.3.1 Screw the ISCA (Figure 3.A) to piece 9 of the enclosure (**Figure 2.K and 3.B**)."*

6. L297-298: Add helpful hints of how to remove air, if it becomes trapped in the enclosure.

We thank the Reviewer for pointing this out. We have added the following note at Lines 351-352: "NOTE: In case some air bubbles are trapped, tilt the enclosure gently with the vent hole facing upward, which will enable the bubbles to escape."

7. L360: Explain how the cultures were enriched for motility or cite an appropriate reference

We have modified the text Lines 426-428 to clarify this point: "To perform the laboratory tests, seawater communities sampled from coastal water in Sydney, Australia, were enriched for motile cells through a simple nutrient amendment (Smriga et al., 2016), as described in step 1.4."

8. Fig 1: It looks like the glue is applied around the entire perimeter of section 1. If you wait a minute after placing part 2a, won't the rest of the glue dry before you have time to place the other pieces (3a, 2b, and 3b) around the perimeter?

The Reviewer is correct, the glue is applied around the entire perimeter of the piece 1 at the beginning. The glue starts to solidify after one minute, but will not be dry yet. This short time only enables us to leave

(i.e) piece 2a stand straight, while placing piece 3a. For more clarity, we have altered the text at Lines 468-469:" The glue takes about 1 min to begin solidifying and allows piece 2a to support itself while placing the next element."

9. L 405-406: The shapes shown in Fig. Ia and Ib are rectangles, not triangles.

*We thank the Reviewer for noting this mistake. We have edited the text at Lines 483-486: "**I.a and I.b.** Glue together two large rectangles (7) and separately glue two smaller ones (8). Repeat once for each. **J.** Glue the four assembled rectangles in the center of the enclosure's lower surface (1)."*