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)."*