Response to Reviewer report:

General Responses (GR):

GR-1: Thanks to the reviewers for their comments. Following their input, the manuscript has been revised. Please see below to read our full response to each of the reviewer’s points.

GR-2: In regards to the justification of for a communication, we invite the reviewers/editors to review our cover letter. Here we highlight/repeat that we chose a communication to rapidly disseminate three discoveries that challenge the conventional wisdom about electroactive biofilms at low concentration: (i) flow reactors can maintain full biofilm activity at concentrations as low as 15 µM (50 times lower than in bulk); (ii) a previously unreported “pseudo-active” metabolic state separates active and inactive states; and (iii) molecular conversion efficiencies can be as high as 90 percent for active biofilms exposed to acetate concentrations at the active/pseudo-activity threshold. We believe that each of these discoveries will require at least one follow up full length paper, which we are working on now. Therefore, this paper and the supporting information focuses on detailed characterization of these phenomena.

GR-3: The manuscript has been entirely reviewed to find and fix typos, etc., including those which were identified by reviewers and others.

GR-4: All new or ammended text is highlighted in yellow, both here in this document and in the revised paper/ESI. In many cases, however, corrections to small typos are not highlighted.

GR-5: The abstract image provided was sized 10 times larger (in both dimensions) than the target dimensions (8x4) to maintain a high file resolution.

Referee: 1

Comment 1-1: In this manuscript, Zarabadi and colleagues present a microfluidic system to measure the electroactivity of Geobacter biofilms. The study uses controls of nutrient concentration and flow to describe various transitional activity states.

Response 1-1: This is point (ii) mentioned in GR-2, above. In addition, two other discoveries were made (points (i) and (iii) in GR-2) as well as the new capability to toggle between activity states.

Comment 1-2: While the study poses some interesting questions, the study is routine in that it presents a number of observations rather than investigate the mechanisms/causes behind these observations. One of the main conclusions is that the nutrient loading rate has an effect on the metabolic activity of the biofilm; this has been know for ~90 years.

Response 1-2: In fact, our (partial) focus, was not simply on the metabolic activity, but on identifying and manipulating the biofilm into fundamentally different metabolic activity states (active, pseudo-active, and inactive). Still, we appreciate the reviewer’s idea about the historical context. We think that it can be useful to add the following passage to connect our work with...
pioneering work of the likes of Monod and C.B. van Niel from the 1940’s on planktonic bacteria and work in the 1970’s & 80’s on biofilms.

“In the 1940’s, Monod and others studied the effect of external influences such as nutrient concentrations on bacterial growth and metabolic activity.\textsuperscript{15} Forty years later, detailed studies into nutrient mass transfer into biofilms and their effects on metabolism were conducted.\textsuperscript{16} However, the difficulty in obtaining sufficient control over reaction parameters continues to limit studies of biofilm metabolic activity at low concentrations.”

Comment 1-3: The one key finding of a pseudo-activity transitional metabolic state is indeed of interest, but other than observing it, the authors make little or no effort to investigate and explain why this is occurring. Accordingly, this preliminary work does not warrant publication in its current form nor as a communication.

Response 1-3: Thank you for your comment. We have four points in response: (1) We wish also to highlight that the independent control over both flow rate and the applied solution concentration enabled us to identify that the main driver in establishing threshold conditions was the convective flux. See Comment 3-3 below on this point. (2) Please see Supporting Information Section 7 (and in particular Figure S4 and Table S2) and the relevant paragraph in the main paper describing a detailed evaluation of the pseudo-active current fluctuations (using FFT power spectrum analysis and statistical methods) to compare and contrast with baseline currents in the active metabolic state. (4) Lastly please see GR-2 about the other key findings and the justification for a communication as a means to communicate the three key findings in advance of detailed work that we are currently undertaking to thoroughly study these phenomena. For example there is discussion and calculations in the paper and SI (Section 8) about the conversion efficiency and its implication to applications in bioelectrochemical systems.

Final comment from Reviewer 1: The authors have addressed all of my comments and questions to a satisfactory level. I therefore recommend publication of this Communication.

Referee: 2

This is an excellent manuscript. Microfluidic bioelectrochemical devices may not be new, but the authors convincingly show the suitability for studying the metabolic properties – here for the examples of low substrate concentrations. The setup works well, the data are clear and are clearly presented.

Response 2-1: N/A

Comment 2-2: There is one point that I would like the authors to address/elaborate: microfluidic systems usually have the problem that oxygen intrusion is difficult to prevent. This makes it difficult to study pure Geobacter cultures (whereas mixed cultures with facultative anaerobes are less of a problem). Thus, I would appreciate if the authors could elaborate the main measures for an oxygen-tight setup.
Response 2-2: Please refer to Supporting Information Section 1 to read about the anaerobic enclosure that was used to prevent O$_2$ penetration through the PDMS microchannel. In addition we have added the following text (to the same SI section) to explain experiments that validate the quality of the anaerobic setup. To validate the ability of the setup to maintain anaerobic conditions during operation, an oxygen indicator (resazurin) was used based on the standard protocol.[1] The resazurin solution flowed into the jar, through the microfluidic device and off-chip into the waste container within the anaerobic jar. This experiment was conducted over 2 weeks without any indication of solution oxygenation. The high electrical current densities produced by the mature Geobacter biofilm matched the upper limits shown in the literature, thus supporting the conclusion that optimal anaerobic conditions were achieved.

Final comment from Reviewer 2: The authors have addressed all of my comments and questions to a satisfactory level. I therefore recommend publication of this Communication.

Referee: 3

Comment 3-1: Chemostats are traditionally used for the controlled growth of microbes in planktonic form, not in biofilm form. The only system that allows this to happen is a microbial fuel cell, if operated on continuous flow. This is an important point that needs to be clarified.

Response 3-1: Thank you for the opportunity to clarify. The reviewer is right that a chemostat is usually used in conjunction with planktonic bacteria, but examples can be found where they supply a constant supply biofilm reactors with a homogenized nutrient solution at user selected conditions.$^{1,2}$ However, we do agree these examples are not typical. Therefore, have removed the word “chemostat” from the paper. For example, the highlighted sentence by the reviewer (i) “To avoid concentration cycling, chemostats can be used to provide a continuous nutrient supply.” has been changed to “To avoid concentration cycling, flow systems can be used to provide a continuous nutrient supply” and (ii) “A centimetre-scale chemostat demonstrated the potential for a flow-based approach to current generation at low concentrations.” Has been change to “A centimetre-scale flow system demonstrated the potential for studies of current generation at low concentrations.”

Comment 3-2: Why? There are plenty of examples of millifluidic MFC systems implemented successfully. In fact one particular study reported on the switching between different substrates and demonstrated the robust ability of the biofilms under continuous flow to toggle in terms of performance. This is pertinent to this study but is missing from the references and should be added:

(In relation to the reviewer’s focus on the underlined parts of the following sentences: “For example, respirometric studies of Pseudomonas biofilms using CO$_2$ permeable silicon tubing$^{24-29}$ could in principle be adapted for electrode-adhered anaerobic EABs, but the integration of electrodes would present a major challenge. Millifluidic systems and larger also suffer from a need for large solution volumes, which pose serious practical problems.”)
**Response 3-2:** We believe that this passage needs clarification. Our goal was to make two separate points: (i) the first was that integrating electrodes into the CO$_2$ permeable silicon tubing used in refs 24-29 would be a major challenge. And (ii) second being that millifluidic channels would consume large amounts of liquids.

In regards to the first point we made a small modification to the yellow sentence: “Millifluidic systems can be easily constructed using standard machining techniques or even with standard flexible tubing. For example, respirometric studies of *Pseudomonas* biofilms using gas-permeable silicon tubing and a CO$_2$ detection system$^{26-31}$ could in principle be adapted for electrode-adhered anaerobic EABs, but the integration of electrodes within the tubing interior and the requirement of an oxygen-purged environment would present difficult challenges.”

Regarding the second sentence, we agree that this point needs clarification. We should have said to obtain flow velocities and shear stress values similar to typical values we obtained in microfluidic channels, large liquid volumes would be required. For example in a millifluidic device that the reviewer was probably referring to (J. You), flow-based MFCs with 25 mm diameter were used. Based on the difference in cross-sectional area between our device and theirs (2500 times), volumetric flow rates would have to be increased 2500 times to obtain the same flow velocity and by much more than that to obtain the same shear stress (see equations S1 and S2 and Table S1 in Supporting information). Also, handling these volumes could only be feasible using peristaltic pumps which do not provide the same flow stability as a syringe pump.

Therefore, we changed the sentence: “Millifluidic systems and larger also suffer from a need for large solution volumes, which pose serious practical problems. Alternatively, microfluidic approaches combine the advantages of low material consumption, established methods...” to “Millifluidic systems and larger must also balance between reasonable solution volumes and limitations to attainable flow velocities and applied shear stresses. Alternatively, microfluidic approaches combine the advantages of low material consumption over a large range of applied hydrodynamic conditions, established methods...”

**Comment 3-3:** Very good!

(In relation to: “To better understand the role of flow in determining the loss of full metabolic activity, we converted the threshold concentration [Ac]$_a$ and the corresponding flow conditions to the threshold convective acetate flux through the channel...”)

**Response 3-3:** N/A

**Comment 3-4a:** This setup is very similar to the one described in: Blauert F. et al. 2015. Biotechnology & Bioengineering, 112, pp. 1893-1905 and although the two lines of work have their differences, it would be worth citing it here as it of similar architecture.

(In relation to Figure 1a).
Comment 3-4b: This is where the paper mentioned earlier (Blauert F. et al. 2015. Biotechnology & Bioengineering, 112, pp. 1893-1905) would be most relevant.

(In relation to: “According to the SEM results discussed earlier, the effect of reduced headspace above the electrode-adhered biofilm resulted in local velocities impinging on the upstream side of the biofilm being 18.7 pmol·s⁻¹·mm⁻².”)

Response 3-4: New text and references are added: “However, both EAB and non-electroactive biofilm thicknesses are known to increase under imposed fluid flow shear, which could affect local flow velocity.⁵²,⁵³ According to the SEM results discussed earlier, the reduced headspace above the EAB, resulted in local velocities impinging on the upstream side of the biofilm being 18.7 pmol·s⁻¹·mm⁻².”

Comment 3-5: The paper mentioned above (J You et al. 2015 Sensing and Bio-sensing Research 6, pp. 43-50) would be most relevant to Fig.2a.

Response 3-5: We think this can be a nice way to compare and contrast our results with others. We have amended the following sentence discussing Figure 2a with a reference to the paper (ref 50):

Unlike in other studies where current decreased monotonically to zero after applying zero concentration nutrient solutions,⁵⁸,⁵⁹ or where a new lag phase was induced after a switching acetate for a complex carbon source,⁶⁰ the switch to the low, but non-zero acetate concentrations used here, induced a fluctuating current (region II). The fluctuating state could be maintained for up to 40 hours, as long as very low, but non-zero concentrations were applied (Supporting Information, Figure S5c).

Comment 3-6: When it comes to flow rate vs growth rate and correlation to power output (as well as COD reduction) again there is literature published using MFCs inoculated with Shewanella oneidensis and operated under continuous flow conditions:

(In relation to the sentence “Figure 3 summarises the results from the experiments described above.”)

Response 3-6: We agree, and have added a paper (new ref 49), which shows a figure with current changing as flow or concentration were changed. As this was an older paper (2011), with the first demonstration of a membraneless microfluidic MFC (using both Geobacter and Shewanella) the effect was not deeply studied, but its Figure 3 is relevant in that it shows how current is affected by flow rate (on or off) as well as changes to concentration in the high concentration regime (0, 5, 10, 20 mM). See new text in Response 3-5. Note this reference was applied to text describing our Figure 2 and not Figure 3, because the latter maps out the metabolic activity regimes whereas the papers and the reviewer’s comments deal with current output (and COD removal).
Comment 3-7: The reviewer did not like the words (a) “material” (“This is clearly the wrong term for a lifeform aggregate. Consider something along these lines (i.e. “lifeform” or “aggregate”).”); (b) “indefinitely” (“you have used this term before, which is unrealistic – suggest to rephrase.”); (c) “tempting” (“This is not a scientific terminology – suggest to rephrase”); and (d) certain typos.

Response 3-7(a): We have changed two instances where “material” was used:

1) Old sentence: “Electroactive biofilms are under intense scrutiny due to their potential as new sustainable materials for bioenergy applications.” New sentence: “Electroactive biofilms are under intense scrutiny due to their potential to enable new sustainable technologies for energy production and bioremediation.”

2) Old sentence: “Electroactive biofilms (EABs) have been heralded as new materials for new sustainable processes...” New sentence: “Electroactive biofilms (EABs) have the potential to accelerate the development of new sustainable processes...”

Response 3-7(b): We have removed the word “indefinitely” and replaced with a more accurate information. Two sentences were changed:

1) Old sentence: “In this manner, rapid and precise changes in both [Ac] and Q could be generated on demand in a matter of seconds and indefinitely maintained.” New sentence: “In this manner, rapid and precise changes in both [Ac] and Q could be generated on demand within a matter of seconds and maintained for durations ranging from seconds to tens of hours”

2) Old sentence: “Interestingly, continued exposure to these conditions resulted in a fluctuating current (region II), which could be maintained indefinitely (Supporting Information, Figure S5c).” New sentence: “The fluctuating state could be maintained for up to 40 hours, as long as such very low, but non-zero concentrations were applied (Supporting Information, Figure S5c).”

Response 3-7(c): We removed the word “tempting” and reworded. Old sentence: “While it is tempting to assume that the relatively low ε_\text{Ac} at high Q is related to reduced contact time between the biofilm and the flowing liquid, this does not explain...” New sentence: “We do not believe that relatively low ε_\text{Ac} at high Q is related to reduced contact time between the biofilm and the flowing liquid, as this would not explain...”

Response 3-7(d): Typos identified by the reviewer (Primareily (Primarily) / Change (changes)) have been fixed along with others. (See GR-3 and GR-4).

Comment 3-8: Based on the comments made above and the missing literature, this sentence is expected to be rephrased accordingly.

(In relation to the sentence: “This work paves the way for new flow-based approaches to optimisation and study of bioelectrochemical systems involving Geobacter and other electroactive biofilms.”)

Response 3-8: We changed the sentence. “Therefore, by extending previous flow studies to microscale flows of low concentration acetate solutions, this work paves the way for new
approaches to optimisation and study of bioelectrochemical systems involving *Geobacter* and other electroactive biofilms.”

**Final comment from Reviewer 3:** The Authors have sufficiently addressed all the comments raised in the original review, so the manuscript is recommended for publication.

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