Reviews

Reviewed by anonymous reviewer, 11 Apr 2023 09:47

Axel Rous et al. submitted a manuscript entitled “Comparison of enrichment methods for efficient nitrogen fixation on a biocathode”. In this study the authors generate H2 in electrochemical cells which can then be used as electron donor to a mix-community of microbes performing a mix of reactions, including acetogenesis via CO2 reduction and N2 fixation.

This is a very complex system with mixed communities where a lot of microbial metabolisms can occur, and where the latter can interact between each other The fact that the medium in not strictly anaerobic also complexifies interpretations. As such, I found that too many statements were substantially speculative. I would also share my concern over the title and abstract of the manuscript, which somehow struggle to share clear take-home messages with the reader.

Below a list of concerns, questions and remarks:

1) Abstract: “These results confirm the enrichment of autotrophic, electrotrophic and diazotrophic bacteria in the polarized cathode enrichments.” Once there is no more organics, only autotrophic microbes would be expected to possibly grow, isn’t it? So no big surprise there. For “electrotrophic”, the authors bring no proof of such phenomenon (an increase in current density is not enough), and the corresponding discussions are purely speculative.

Thank you for this comment, we agree that the previous formulation of the abstract might not have accurately represented the findings of our study. It has been revised to provide a more balanced and accurate representation of our results.

Regarding the term "electrotrophic," it is true that there is an ambiguity in our discussions. We choose to use the term "autotrophic" to refer to microorganisms that can utilize a cathode as an energy source, either through direct electron transfer or through mediated electron transfer (potentially using hydrogen as a mediator).

The abstract has therefore been modified as follows (l. 16-l. 38):

“The production of nitrogen fertilizers in modern agriculture is mostly based on the Haber-Bosch process, representing nearly 2% of the total energy consumed in the world. Low-energy bioelectrochemical fixation of N_2 to microbial biomass was previously observed but the mechanisms of microbial interactions in N_2-fixing electroactive biofilms are still poorly understood. The present study aims to develop a new method of enrichment of autotrophic and diazotrophic bacteria from soil samples with a better electron source availability than using H_2 alone. The enrichment method was based on a multi-stage procedure. The first enrichment step was specifically designed for the selection of N_2-fixing bacteria from soil samples with organic C as electron and carbon source. Then, a polarized cathode was used for the enrichment of autotrophic bacteria using H_2 (hydrogenotrophic) or the cathode as electron source. This enrichment was compared with an enrichment culture of pure diazotrophic hydrogenotrophic bacteria without the use of a microbial electrochemical system. Interestingly, both methods showed comparable results for N_2 fixation rates at day 340 of the enrichment with an estimated average of approximately 0.2 mgN_{fixed}/L.d. Current densities up to -15 A/m² were observed in the polarized cathode enrichments and a significant increase of the microbial biomass on the cathode was shown between 132 and 214 days of enrichment.These results

confirmed an enrichment in autotrophic and diazotrophic bacteria on the polarized cathode. It was hypothesied that autotrophic bacteria were able to use either the H_2 produced at the cathode or directly the cathode through direct electron transfer (DET) as more biomass was produced than with H_2 alone. Finally, the analysis of the enriched communities suggested that Desulforamulus ruminis mediated microbial interactions between autotrophic anaerobic and heterotrophic aerobic bacteria in polarized cathode enrichment. These interactions could play a key role in the development of biomass in these systems and on N_2 fixation. Based on these findings, a conceptual model on the functioning of mixed cultures N_2-fixing electroactive biofilms was proposed."

2) l. 181: you flush the electrochemical cell when there is more than 10% then 5% of O2 in the gas phase. This is very surprising to perform (bio)electrochemical experiments at such low potential (-0.94 V vs SCE) under the presence of O2. At circumneutral pH, O2 get readily reduced to H2O2 below around -0.3 V vs SCE on conventional carbon electrodes. It would therefore impact the current and generate trace amount of H2O2. The authors should discuss that probable reaction and the corresponding implications.

Thank you for raising this point as it highlights an important aspect that warrants further discussion. The enrichment procedure used was inspired by the work of Rago *et al.* (2019) who investigated microbial development on a cathode poised at -0.7 V vs. SHE under two conditions: exposure to air and exposure to anaerobic conditions (Rago et al., 2019). They demonstrated a more significant microbial growth on the cathode in the presence of oxygen despite a potential production of hydrogen peroxide (with a pH around 8). Considering these observations, we did not consider the production of hydrogen peroxide but this aspect is now discussed in the revised version of the paper.

The following statement has been added in result and discussion section about coulombic efficiencies (l. 555-l. 563):

"In addition, in presence of O_2 in the cathodic chamber, oxygen reduction reactions were expected with regards to the potential used in this study. Indeed, a two-electron reduction could have occurred, resulting in the production of hydrogen peroxide (H_2O_2), which can then undergo further reduction to form water (H_2O) (Rozendal et al., 2009; Sim et al., 2015). Hydrogen peroxide was not measured, however, the amount of biomass found on the cathodes (Figure 4) suggested that the concentrations of hydrogen peroxide were sufficiently low to have minimal to no impact on the microbial community during the enrichment process. Nonetheless, a fraction of the electrons may have been lost through these oxygen reduction reactions, which could partially account for the low coulombic efficiencies observed in this study."

3) I think Fig. 1 means that there is no nitrogen fixation for nPCE control. However, ammonium production rate for nPCE is the highest in Table 1 (0.07 mg/(L.d) N-NH4, equivalent to 23 mg/L of N-NH4 over the 340 days of the experiment. The authors claim that this NH4 is generated via cell lysis. I am very confused about the fact that a negative control (without supplying electron donor) would generate a product of interest for this study (NH4+) at the highest rate. Could the authors further discuss this result?

The confusion regarding the level of ammonium in the negative control (nPCE) can be attributed to a lack of clarity in the description of the enrichment, which led to a misunderstanding. nPCE and PCE were both enriched using organic carbon in a first phase before transitioning to using only CO_2 as carbon source (see revised Figure 1). This particular step of the enrichment process facilitated a biomass increase as evidenced by qPCR analyses (see biomass estimated at day 125 in Figure 4).

Material and methods was clarified, a figure describing the different enrichment steps for the different reactors has been added.

An explanation was also added in the first part of the Results and discussion section (l. 397-l. 402):

"This higher biomass concentration was probably due to the presence of organic C (day 0 to 115 including 60 days in BES) for the PCE and nPCE enrichments. NH_4^+ in nPCE was also observed at a rate of 0.07 mg/L.d as presented in table 2 but without acetylene accumulation, meaning that no N_2 fixation occurred. In absence of electron sources, nPCE enrichment communities was therefore only maintained through a cryptic growth. The presence of NH_4^+ in the nPCE was likely related to cell lysis since no measurable fixation was detected by ARA even though significant biomass production was observed."

And the following statement was also added in the Results and discussion section, in the paragraph dealing with N quantification and CE (l. 521-l. 527):

"Using our method of N mass estimation on biomass lost as presented in figure 3 for nPCE, the total concentration of N lost by biomass would be estimated around 130 to 200 mg_N/L depending on the community (2 nPCE). This loss would then be equivalent to rates of 0.6 to 0.9 mg_N/L.d released by this the biomass on average along the enrichment. Assuming a loss of a constant portion of biomass, a release rate of 0.1 mg_N/L.d was estimated as average for the batch ending at 340 days, close to the 0.07 mg/L.d presented in ***Erreur ! Source du renvoi introuvable.****. This result supported our hypothesis that NH_4^+ release was linked to biomass loss in nPCE enrichments after organic C addition was stopped."*

4) l.345 Similarly to previous comment: "In nPCE controls, the biomass was higher than in H2 enrichment". In Methods, it is stated that the electrolyte used in the electrochemical cells is "the same inorganic medium", which suggests it was the N-free medium (this should be clear, not suggested). How can biomass grow without any assimilable nitrogen (e.g. ammonium, nitrate) and without ability to fix nitrogen (see Figure 1)? How come it can grow more than the community specifically enriched to fix nitrogen?

Thank you for this comment. The problem pointed here comes from the lack of clarity in the description of the steps of the enrichment procedures as mentioned in our previous answer.

We believe that the modifications described above correctly address this issue and will provide a clearer and more accurate description of the experimental procedure.

5) l. 358: "Regarding the current densities in the abiotic systems, the average current densities were measured at -0.75 A/m2 for two times four days with an organic C source

and -1.1 A/m2 for 16 days with only CO2 as the carbon source." This is unclear, do the author mean they only periodically recorded the abiotic current? This would make this control irrelevant. A rigorous control should be continuously polarized during the whole experiment, which is very long (> 300 d). The cathode has all the time to be modified because of the polarization, for example by reducing trace elements on the carbon surface that would catalyze H2 evolution (or O2 reduction, another issue previously mentioned) and increase abiotic current over time.

Indeed, the current densities of abiotic controls were measured in short experiments. Obviously, a 300-day abiotic control following the same procedure as the enrichments, including the use of a rich medium with organic carbon for 60 days followed by a switch to CO_2 as the sole carbon source, would have allowed a more comprehensive monitoring of abiotic reactions. However, maintaining sterile conditions throughout such a long-term control would have been a significant technical challenge. We thus opted to prioritize Open Circuit Voltage (OCV) controls (nPCE) throughout the enrichment process with biological duplicates, rather than employing long-term abiotic electrolysis cells. Despite the limitations arising from the absence of a continuous, long-term abiotic control, we believe the maximum current densities observed in short term abiotic conditions presented in Figure 3 still provide relevant insights into the electrochemical performance of the biotic system in comparison with abiotic systems.

The following sentences have been added in the Material and Method section to be clearer on the use of abiotic reactors and the duration of the measurement (l. 162-l. 165).

"In order to validate the role of the cathode as electron source, two OCV (nPCE) reactors were carried out. The current densities of the abiotics reactors over a short period were therefore used as a reference to be compared with the measurements made after inoculation and monitor the increase of the activity of reduction at the cathode. "

6) l. 370: "the current [was] likely due to the direct use of electrons by bacteria in a cathodic biofilm as proposed by Z. Zaybak et al." At this potential it is common knowledge that (i) H2 evolution occurs; (ii) O2 reduction occurs. The aforementioned statement is therefore extremely speculative. In fully anoxic conditions, the recording of polarization curves could possibly suggest a DET phenomenon if they would exhibit a clear sigmoidal curve that is typical for microbial electrocatalysis. However, the absence of cyclic voltammogram in the manuscript (or SI) makes it impossible to suggest such a microbial electrochemical process.

We agree that the statement was rather speculative. We have thus revised the sentence as follows in the first Results and discussion paragraphs (l. 423-l. 426):

"The high current density observed after 250 days of enrichment indicated a high redox activity linked either to hydrogen evolution, oxygen reduction, or possibly direct electron transfer. As proposed by Z. Zaybak et al. (2013), the high activity was probably resulting from a high metabolic activity in the biofilm with significant microbial catabolic process (Zaybak et al., 2013). "

7) Often, in research related to BES, one would perform basic controls to assess which molecule(s) is involved in the generation of current before making any claim. Here multiple compounds could be related to abiotic and biotic current generation (e.g. reductions of O2, N2, CO2, nitrate and obviously protons to H2). Here I miss relevant tests screening the impact of those different compounds, making any interpretation of the overall electron flow very speculative.

Thank you for this feedback, we acknowledge that various compounds, including oxygen, nitrogen, carbon dioxide, nitrate, and protons, could have potentially contributed to both abiotic and biotic current generation in our systems. In this study, the main focus was to track the enrichment process over time and investigate microbiological aspects linked with the nitrogen fixation process, we thus didn't explore this aspect.

The experiments were long and delicate and performing a comprehensive screening with different potential electron acceptors during the experiments could have compromised the viability or the activity of the biofilms. We agree that further investigations are needed to explore the electron transfer mechanisms with more targeted experiments, this will be the objective of future experiments. The following sentences have been added at the conclusion of the main text (l. 712-l. 716):

"In order to focus on the enriched microbial community and avoid to disrupt the enrichments, a comprehensive screening with different potential electron acceptors was not performed here. However, such screening would be interesting to be investigated and could be the subject of further studies on synthetic community to further explore the impact of electron acceptors on microbial communities, current densities and coulombic efficiencies."

8) Reported coulombic efficiency range from 9% to 63% depending on replicates and period of study. Where are going the other electrons?

In microbial electrochemical reactors, determining the precise distribution and fate of electrons is challenging as highlighted by the comprehensive work of de et al. (2022), who compiled results from two decades of experiments with microbial electrochemical reactors for wastewater treatment and reported a median coulombic efficiency of 20% (de Fouchécour et al., 2022). In our study, the unaccounted electrons could potentially be associated with oxygen reduction reactions or unfollowed biologic reactions as exopolysaccharide (EPS) production, contributing to the observed variability in coulombic efficiencies. These aspects are now explained in the main text (l. 577-l. 581).

"As seen in Table 3, electrons were distributed in biomass production, N_2 fixation products, H_2 accumulated in headspace and in CH_3COOH produced from CO_2. However, these reaction products do not allow the electron balance to be completed. The electron losses and the differences observed between the cathodes in PCE1 and PCE 2 were explained by side reactions, such as O_2 reduction or biologic reaction as exopolysaccharide (EPS) production."

9) Communities from one replicate to another can vary to a very large extent, yet here only one “average relative abundance” is provided per enrichment in Fig. 5. I think the Methods section does not provide information on the number of replicates tested. Exhibiting only one result per enrichment would not be very relevant. If several setups were tested for communities, the respective data per setup should be presented.

Microbial analysis was carried out in all available reactors, consisting of four bottles for each pre-enrichment, six bottles of hydrogen fed enrichments (H_2E) and two reactors for each PCE and nPCE experiments. Summarizing the microbial analyses for all these reactors posed a significant challenge, and we acknowledge that the previous figure did not provide an optimal solution. Therefore, we decided to employ Principal Component Analysis (PCA) to provide a more comprehensive representation of the microbial communities. This representation was made using families of the five major OTU of each sample at the time of sequencing. As a complement, barplots of bacterial families relative abundances are provided in supplementary material.

The following elements were added in the result and discussion section (Microbial Community) (l. 594-l. 636, l. 641-l. 643, l. 660-l. 664):

Figure 1 *– Results of the principal component analysis (PCA) performed on the microbial communities of a) pre-enrichments, H_2-fed enrichment bottles (H_2E) after 232 days and cathodes of PCE and nPCE enrichments after 214 days and b) H_2-fed enrichment bottles (H_2E) after 232 days and cathodes of PCE and nPCE enrichments after 214 days. Only families of the five major bacterial OTU in each sampled community were used for the analysis. The microbial communities in the pre-enrichment bottles are represented by the following abbreviations: F for forest soil, C for compost, L for the rhizosphere of leguminous plants, and M for a mix of all. Variables least close to the correlation circle are not displayed (cos2 < 0.2).*

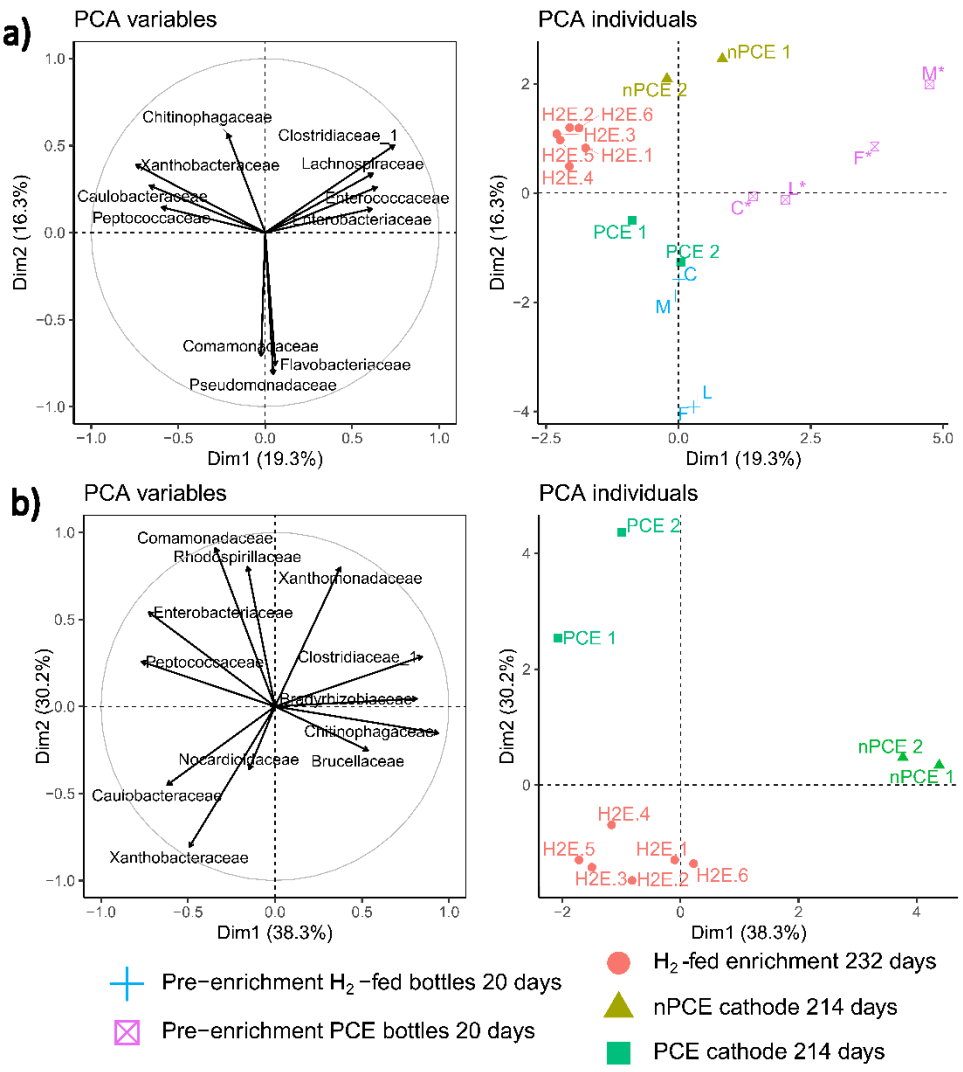
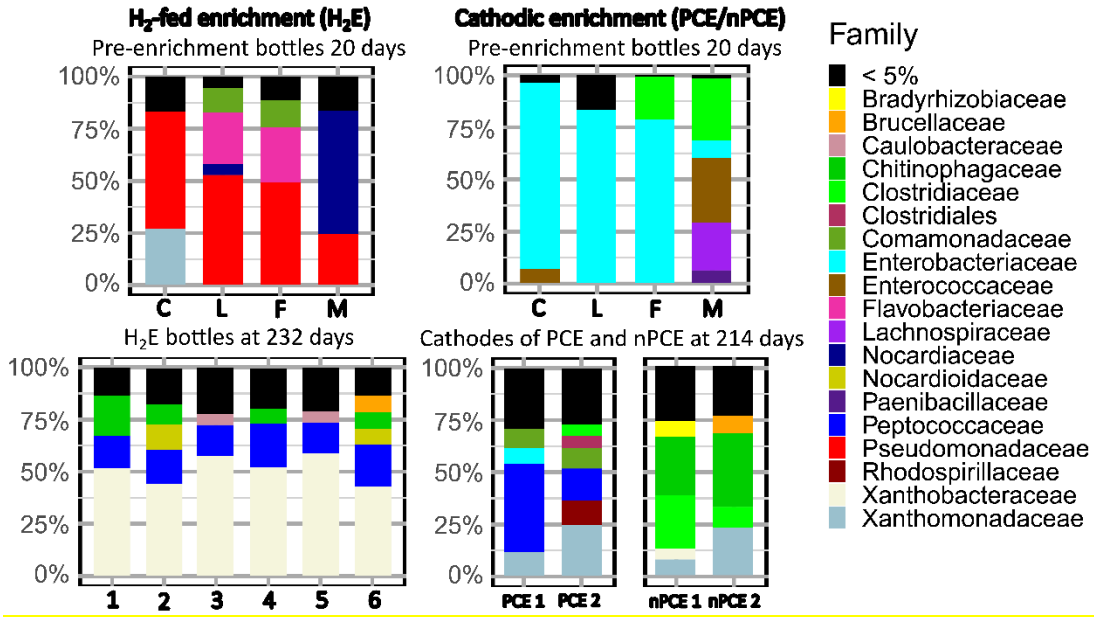


Figure 7 *– Barplots of relative abundances of major bacterial families of pre-enrichments, of H_2-fed enrichment (H_2E after 232 days) and cathodic enrichment (PCE and nPCE after 214 days). The microbial communities in the pre-enrichment bottles are represented by the following abbreviations: F for forest soil, C for compost, L for the rhizosphere of leguminous plants, and M for a mix of all. Only families with a relative abundance ≥ 5% are shown for each sample.*



A paragraph has also been added in discussion section about community analysis:

"16S rDNA sequencing was performed at the end of pre-enrichment , and at 214 or 232 days of enrichment in polarized cathode enrichment (PCE) and in H_2-fed enrichment bottles (H_2E), respectively. The sampling days were selected because they were associated to a high microbial activity (high current densities and high biomass concentrations). In H_2E, the nifH/16S abundance ratio was also maximum (0.9) at day 232. Principal Component Analysis (PCA) was used to present the communities for each enrichment. Each reactors and bottles are presented as individuals and major families as variables in PCA presented in the Figure 1 and relative abundances are presented in the Figure 7.

For communities at the end of the pre-enrichment, the principal component analysis (PCA) showed an important link between the families of the Clostridiaceae, Enterobacteriaceae, Enterococcaceae and Lachnospiraceae and with the PCE pre-enrichment (Figure 6a). Indeed, the group of communities of pre-enrichment with organic C is well separated from the others groups and follow the same direction as these four families. A dominance of the Enterobacteriaceae family (mainly of the Citrobacter genus) was observed for each pre-enriched sample expect for the pre-mixed sample where the three others families are highly present. These families are therefore absent or very weakly represented in the other sequenced communities as shown in Figure 7.

For the H_2-fed bottle pre-enrichments (H_2E), the group is also seprated of the 232-days enriched community H_2E. Pseudomonadaceae (45% of average relative abundance) family was mostly dominant at the end of pre-enrichment. Nocardiaceae, Flavobacteriales, Xanthomonadaceae and Comamonadaceae were also present as seen in ***Erreur ! Source du renvoi introuvable.****. Flavobacteriales and Comamonadaceae families are also higly linked with the group of pre-enrichment in PCA of Figure 1a. These families, with the exception of Flavobacteriales, are also known to have members possessing the set of genes necessary for N_2 fixation (Dos Santos et al., 2012; Ghodhbane-Gtari et al., 2019; Huda et al., 2022). These families accounted for 77% of the sequences which is high compared to the nifH/16S rDNA ratio of less than 0.01 at the same time point. This suggests that either the nifH primers were not adapted to these specific species or that the species found at this point did not possess the genes for nitrogenases. As the*

H_2E pre-enrichment cultures started on a medium containing NH_4Cl, the presence of this source of nitrogen was likely favorable to the growth of non-diazotrophic bacteria.

After 214 days of enrichment, PCE communities were affiliated to Peptococcaceae (29% in average), Xanthomonadaceae (18% in average), Rhodospirillaceae (11% in PCE 2, Azospirillum) and Comamonadaceae (10% in average) as presented in figure 7. As seen in the PCA presented Figure 1b, Rhodospirillaceae, Comamonadaceae, Enterobacteriaceae and Xanthomonadaceae families are representative of the PCE cathode communities. Peptococaccaceae appear to be shared with communities of H_2-fed enrichment (H_2E) bottles. As seen in Figure 1a and ***Erreur ! Source du renvoi introuvable.****, a clear shift in microbial communities from the end of pre-enrichment was therefore observed as the difference between PCE communities at 214 days and at the end of pre-enrichment.*

*The Comamonadaceae as well as the Enterobacteriaceae families mostly include heterotrophic species, which would be consistent with our hypotheses about the existence of interactions between heterotrophic and autotrophic populations (Liu et al., 2011; Wu et al., 2018)"*The *Xanthobacteraceae* (51% in average), *Peptococcaceae* (17%, identified as *Desulforamulus*), *Chitinophagaceae* (8%) and *Nocardioidaceae* (5%) families were found to be dominant in H_2E bottles at day 214 as presented in figure 7. The *Xanthobacteraceae* family, highly linked to H_2E communities as seen in Figure 1b, was mostly represented by the species *Xanthobacter autotrophicus* which is known as N_2-fixing HOB (Wiegel, 2005)

10) Still about communities, you state (l. 525) that "at the beginning of PCE in bottles fed with organic C, communities were strongly dominated by only four families […] This observation indicated a rapid selection of bacteria having the capability of N2 fixation at the early stage of enrichment." This is very unclear since you refer to the results expressed either as "at the beginning of enrichment" or "start (in bottle)". This suggest it is your initial composition of your community, so could it be already "enriched" by a "selection"? If those are not the initial compositions (inocula), where are the latter, since it is obviously necessary to assess an enrichment? Overall the community results are presented in an ambiguous manner.

Thank you for your feedback on this point which was not fully clear. Tio improve the clarity of the text, we are now referring to "pre-enrichment of cultures", as the end of the first batch of each enrichment procedure, as presented in material and methods. In order to clarify this point, the following sentences have been added in the material and method section regarding the enrichment procedures (l. 185-l. 192):

"Each soil sample (Forest, Leguminous and Compost) and a mix of all were used as inoculum in one batch of pre-enrichment. Pre-enrichment cultures were then carried out in the same medium as first enrichment step for each procedure (i.e. supplied with organic C for the first procedure and inorganic medium for the second) and for 20 days prior to be used as inoculum in the enrichment procedures. Samples of the pre-enrichment were considered as initial microbial community of the enrichments. Thus, at the end of pre-enrichment, the mixed culture was used to inoculate two bottles of each enrichment and all unmixed soil samples were mixed to inoculate four bottles of each enrichment."

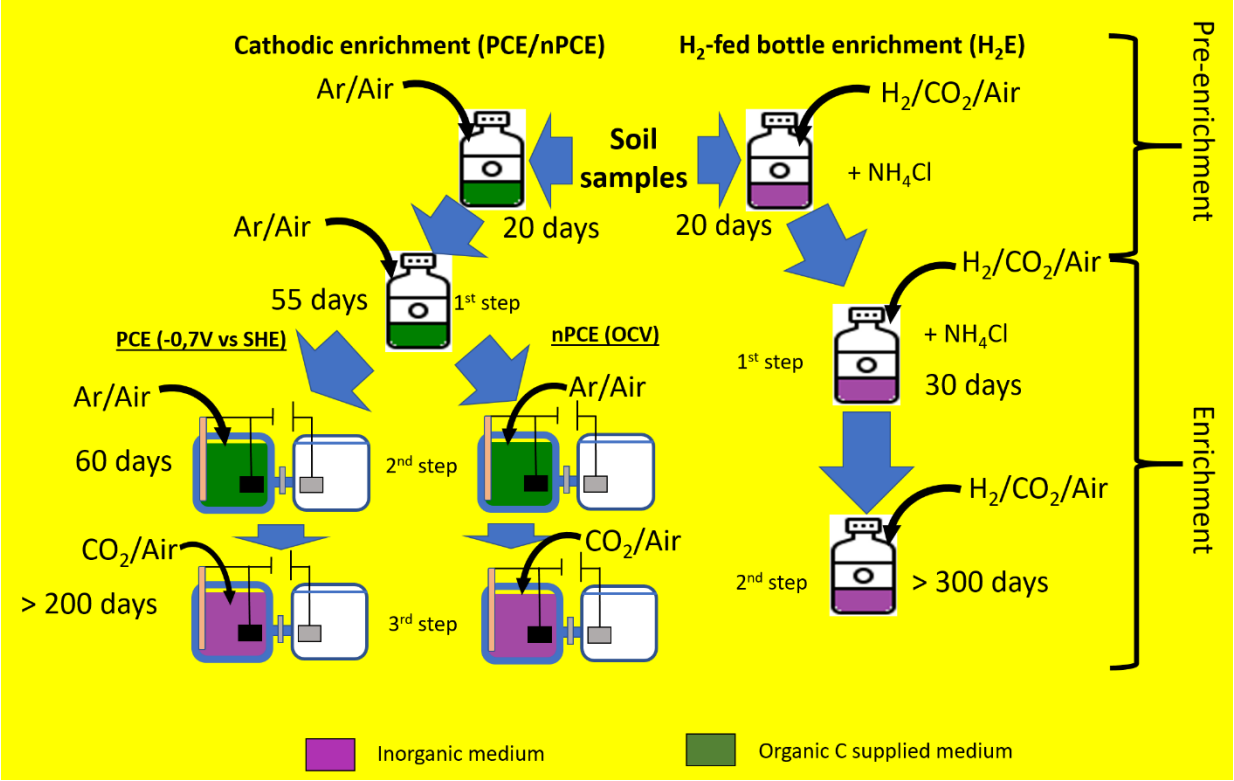
The following sentences have also been added in the Material and method section regarding the sequencing procedure (l. 350–l. 354).

"Communities sequenced on pre-enrichment bottles were used as initial community of the enrichment cultures. For PCE and nPCE, sequenced communities came from the biofilm formed on electrodes. Two replicates per potential were used. For H_2E bottles, 6 bottles were used for sequence analysis and qPCR. For pre-enrichment, sequences corresponded to each soil samples (leguminous, forest, compost) and a mix of them."

Finally, community analysis in result and discussion section has also been modified (l. 626–l. 628).

"As the H_2E pre-enrichment cultures started on a medium containing NH_4Cl, the presence of this source of nitrogen was likely favorable to the growth of non-diazotrophic bacteria."

The following scheme was also added in material and method about the enrichment procedure to add a clearer representation of the different procedures used in this work.



Some details:

1) What is "H2 enrichment"? Please give clear names to your enrichments and use them consistently along the whole manuscript. Now in Method section you name them "first" and "second enrichment", then use acronyms or other terminologies: it is confusing. You may also consider that the electrochemical setup in closed circuit would very likely also induce a H2-enrichement at this low cathodic potential, adding to the confusion.

Thank you for pointing out this potential confusing issue. "H_2-fed enrichment (bottles)" and the acronym "H_2E" were both used to refer to the enrichment procedure where H_2 was externally added. The following indications have been added to clarify this point (l. 193 and l. 219):

" The first enrichment cultures, polarized cathode and non-polarized cathode enrichments (PCE and nPCE) were performed in three steps."

"In the second enrichment method, the H_2-fed enrichment (H_2E), autotrophic bacteria were enriched in inorganic medium supplemented with H_2 as sole electron source. "

2) l. 117: "The vitamin solution consisted of 0.1g ZnSO4 7H2O, 0.03g MnCl2 4H2O, 0.3g H3BO3 0.2 CoCl2 6H2O, 0.01g CuCl2 2H2O, 0.02g NiCl2 6H2O, and 0.03g Na2MoO4 2H2O per liter of solution." Those are not vitamins.

Thank you for this mistake that was corrected as follows (l. 126-l. 130):

"The SL-6 trace element solution consisted of 0.1g $ZnSO_4$ 7H_2O, 0.03g $MnCl_2$ 4H_2O, 0.3g H_3BO_3 0.2 $CoCl_2$ 6H_2O, 0.01g $CuCl_2$ 2H_2O, 0.02g $NiCl_2$ 6H_2O, and 0.03g Na_2MoO_4 2H_2O per liter of solution. The vitamine solution consisted of 10mg Riboflavin, 50mg Thiamine-HCl 2H_2O, 50mg Nicotinic acid, 50mg Pyridoxine-HCl, 50mg Ca-Pantothenate, 0.1mg Biotin, 0.2mg Folic acid and 1mg Vitamin B_{12} for 100 mL of distilled water."

3) l. 142: "The monitoring of the current intensity was used to monitor the efficiency in enriching the biofilms in autotrophic and/or electrotrophic bacteria." How come? At this low potential that can induce abiotic electrochemical reactions, a current alone does not necessary reflect a biological activity.

The sentence was modified as follows to remove the "electrotrophic" term (l. 152-l. 157). Indeed, the current density did not reflect the biological activity but reflected the availability of electron source for autotrophic bacteria. These electron sources are then assumed to be H_2 abiotically produced at the cathode or the cathode can act in direct electron transfer (DET).

"The current intensity was used to monitor the availability of electrons in the electroactive biofilm. An increase of the current intensity was representative of an increase of the reduction reactions at the cathode. The increase of these reduction reactions, abiotic or not, was assumed to bring more electrons to the bacterial community. It was therefore assumed that an increase in current intensity was related to the enrichment of bacteria able to use the cathode as electron source (Zaybak et al., 2013)."

4) l. 150: "The energy required for the production of microbial metabolites and for biomass growth was then used to calculate the Coulombic efficiency". Maybe you mean "the electrons required" since there is no thermodynamic data provided for the chemical reactions stated, and obviously there is no energy consideration for assessing a coulombic efficiency.

Thank you for the comment on terminology. Mentions to "energy" have been replaced by "electron" when necessary. The following sentences have been modified (L.25, l. 113, l. 166, l. 185, l.217, l. 220, l. 369, l. 453, l. 462, l. 515, l. 646, l. 698).

5) l. 164: “the Coulombic efficiency in percentage of electron recovery in circuit”. The equation above means the opposite: recovery of electrons in products.

This terminology replacement has been corrected as follows (l. 180).

“With η_{CE} the Coulombic efficiency in percentage of electron recovery in products”

6) l. 165: “molproduit”

Same as the previous comment.

$mol_{product}$

7) Check the caption of Figure 1, seems there is an issue there.

The captions have all been revised to avoid issues.

8) Caption Table 1: “average ammonium”. I guess “production rate” is missing.

Thank you for the correction of this caption which have been modified as follows:

“Table 2 - The average ammonium production rates, the number of nifH gene copies, the number of 16S gene copies, and the nifH/16S ratio were assessed after 340 days for the three experimental configurations. Average values were measured on the last batch of 21 days, before 340 days of enrichment for the two polarized cathode enrichment (PCE), the two non-polarized Cathode Enrichments (nPCE) and the six H_2 enrichment bottles (H_2E)”

9) l. 364: “with regards to the average current in the abiotic controls and standard deviation up to -2 A/m2”. Unclear.

To make this sentence clearer, we have made the following corrections (l. 416-l. 419).

*“An increase up to -2 A/m² appeared between 100 and 115 days of enrichment in PCE. CO_2 was then used as sole carbon source after this increase appeared. Following this change in carbon source, a sharp increase in current density to -5 A/m² was observed in the PCE with regards to the range of current densities in the abiotic controls (approximately -2 A/m² on CO_2)(**Erreur ! Source du renvoi introuvable.**). “*

10) l.368: “current consumption”. One do not “consume” current.

Thank you for this clarification on the correct formulation to use. “Current density” is now used where “current consumption” was mentioned as seen in the example below:

“The high current density observed after 250 days of enrichment strongly suggests a specific enrichment in electroactive bacteria. Indeed, current density was 5 to 10 times higher than in average for the abiotic controls, and was likely due to the direct use of electrons by bacteria in a cathodic biofilm as proposed by Z. Zaybak et al (Zaybak et al., 2013).”

11) l. 465: “The C/N ratios are shown in Figure 4. C/N ratios in the PCE were around 10 after 131 and 214 days in comparison with ratio of 5 to less than 1 in the nPCE control. These ratios are consistent with the theoretical ratio of 8 to 10 assumed for microbial biomass.” (i) Please read the sentences yourself to assess the obvious discrepancy between both statements; (ii) for day 214 I read more something like 15 +/- 14.

Thank you for this comment. It seems that several words have been forgotten in the sentence, making it difficult to understand. It seems that this part concerning the C/N ratio brings more complexity for the understanding of the subject and this one was thus withdrawn. It did not bring essential information to our conclusions.

12) l.468: so what is the other dry carbon coming from?

Thank you also for this comment which confirms the lack of clarity in this part of the C/N ratios. As mentioned in the previous comment, this one has been removed.

13) Fig. 4 caption: “Total N concentration based on N measured in dry weight from medium samples for polarized cathode enrichment (PCE) and non-polarized cathode enrichment (nPCE) and estimated from biomass for H2-fed enrichment (H2E)”. Do you dry the medium samples and measure N? Do you express it with respect to liter of sample? Then you only “estimate” it for H2 enrichment medium? I find the caption confusing.

This caption has been revised as follows to make it clearer. And to answer the questions, the medium was dried before nitrogen measurement (CHNS analyzer method) and the volume before drying was used to calculate the PCE and nPCE concentration. And about H_2E, the volume was too low to be used in this measurement of N in medium so the total N calculated for H_2E is only based on N estimated from biomass measurement and NH_4^+.

“Figure 5 : Total N concentration based on the sum of (1) N estimated from biomass measurement (suspended biomass and biofilm), (2) N content in ionic forms (N-NH_4^+, N-NO_3, N-NO_2) and (3) N measured in the dry weight of the medium of the polarized cathode enrichment (PCE) and non-polarized cathode enrichment (nPCE) and (b) N-NH_4^+ concentration in H_2 enrichment (H_2E), PCE and nPCE”

14) l.493: “These high coulombic efficiencies were probably also associated to microaerophilic conditions.” I don’t see why the microaerophilic conditions would increase coulombic efficiency for biomass formation, develop your thoughts. Also, the CE is calculated assuming a constant C/N ratio (Eq. 1) while your results show very different and evolving C/N ratio (Fig. 4,c).

Thank you for pointing out this sentence. This assumption was made on the basis of an acetate production from one strain (acetogen) that could be used by another aerobic strain (acetotroph). The high Coulombic efficiencies could therefore be attributed to both acetotrophic and

acetogenic bacterial growth. However, with regards to the complexity of the microbial communities this assumption was rather speculative and we decided to remove this sentence.

Reviewed by anonymous reviewer, 09 Apr 2023 08:05

This study pertains to studying different approaches for the enrichment of N2-CO2 fixing microbial cultures. Such alternate N2 fixation strategies are highly desired. I have the following queries or comments on the methods and results/discussion sections that should be considered for further improvement of the paper.

1. It would be better to provide a clear comparison table for the enrichment methods. This would justify the title of the paper. How these approaches differ from those tested earlier by other researchers should also be stated.

Thank you for this remark. Indeed, a figure with the three different enrichments used here could help in a better understanding of the procedures used. A graphical representation of the enrichments to further improve understanding has been added.

The following diagram and table have been added in the material and method section:

Figure 2 - Diagram of the enrichment process

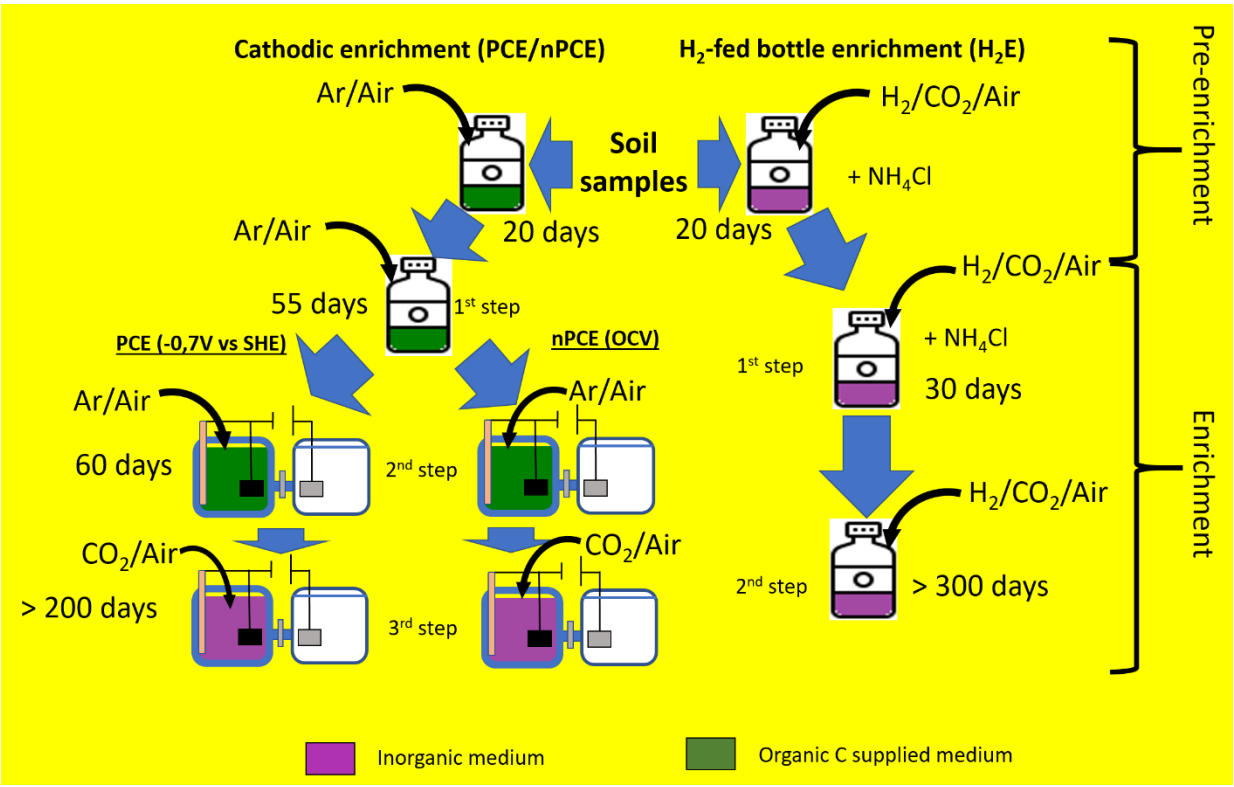


Table 1 - Comparison between each enrichments procedure used in this work.

	PCE	nPCE	H2E

Enrichment reactor	Bottle then cathodic chamber	Bottle then cathodic chamber	Bottle
Electrode	Yes	Yes	No
Applied potential	-0,7 V vs SHE	Open circuit voltage	NA
Enrichment step	3 steps (1) soil diazotrophic bacteria in bottle (2) diazotrophic bacteria in MEC (3) autotrophic diazotrophic bacteria in MEC	3 steps (1) soil diazotrophic bacteria in bottle (2) diazotrophic bacteria in MEC in OCV (3) control without electron sources	2 steps (1) soil autotrophic bacteria in bottle (2) autotrophic diazotrophic bacteria in bottle
Number of Batch	(1) 10 (2) 4 medium replacement (3) 14	(1) 10 (2) 4 medium replacement (3) 14	(1) 2 (2) 25
Batch duration	3-7 days (Organic C) 15-30 days (CO_2)	3-7 days (Organic C) 15-30 days (CO_2)	15-30 days
Enrichment duration	>300 days	> 300 days	> 300 days
Electron donor	(1) (2) Organic C (2) (3) Cathode (direct or indirect)	(1) (2) Organic C (3) none	(1) NH_4Cl and H_2 (2) H_2
Electron acceptor	$O_2/N_2/CO_2$	$O_2/N_2/CO_2$	$O_2/N_2/CO_2$

To answer the second question about the other researchers and our positioning, a short description was added for each works in the presentation of this other works as presented in the next comment.

2. Line 62: The authors have missed referring to some latest studies on this topic. These include (i) Soundararajan et. al., 2019 (https://doi.org/10.3389/fmicb.2019.01817); (ii) Chen et. al., 2020 (https://doi.org/10.1128/AEM.01998-20); (iii) Yadav et. al., 2022 (https://doi.org/10.1016/j.jcou.2022.101997).

Thank you for this comment on literature. Some of these works were performed with pure strain, ie. Soundararajan et al. (2019) and Chen et al. (2020). These studies were omitted on purpose because they were not dealing with enrichment procedures. Nonetheless, we agree that these studies are relevant for the topic of nitrogen fixation in bioelectrochemical system. A sentence was added to improve the discussion. For Yadav et al. (2022), they are cited in comparison with our results. Other works of interest made by Li et al. (2022) and Zhang et al. (2022) were then added to provide a larger overview of the topic as follows:

Presentation of the others works has been added as follow in introduction (l. 65–l. 78):

"Other works investigated the mechanisms with pure bacterial strain like Soundararajan et al. (2019) and Chen et al. (2020) with Rhodopseudomonas palustris and Pseudomonas stutzeri(Chen et al., 2020; Soundararajan et al., 2019). Yadav et al. (2022) demonstrated the possible use of N_2-fixing bacteria as nitrogen source in a microbial electrosynthesis process of acetate(Yadav et al., 2022). Zhang et al. (2022) also worked on a system of N_2 fixation in microbial electrolysis cell (MEC)(Zhang et al., 2022). In these works, the authors investigated the interactions existing between CO_2 and N_2 fixation microbial processes. Coupling capabilities of N_2 fixation in bioelectrochemical systems as possible source of nitrogen for other biological systems was subject of interest. Indeed, such coupling can lead to the production of molecules of interest such as acetate by reducing the environmental impact of the use of reactive nitrogen often in the form of NH_4Cl (Yadav et al., 2022). The work of Li et al. (2022) in a single-chamber system and highlighted an importance of synergy within an N_2-fixing community in a microbial bioremediation system (Li et al., 2022).

All this work has demonstrated that it is possible to use a cathode as an electron source for biomass growth by fixing N_2 and CO_2.."

the following sentences have also been added in the results section to improve the discuss (l. 586–l. 592):

"In comparison with the other works dealing with N_2-fixing cathodic biofilms, Zhang et al (2022) showed a maximum of 40.5 mg/L of NH_4^+ in 4 days with mixed communities, in regard to a maximum of 0.8 mg/L NH_4^+ observed in Yadav et al (2022) and 6.31 mg/L NH_4^+ in 10 days for Li et al. (Li et al., 2022). When demonstrating the N_2 fixation in MES, Rago et al (2019) showed a N_2 fixation of 0.2 mgN/L.d in biomass and 5 10^9 bacteria/L.d. In the present study, biomass production in biofilms was 2 to 10 times higher than in Rago et al. (2019)*, as was the nitrogen found in the biomass varied between 0.2 and 1 mg/L.d before 214 days."*

3. Line 66-69: It should be noted that not only biomass but some organic compounds can also be synthesized through this approach. For example, Yadav et al. 2022 ((https://doi.org/10.1016/j.jcou.2022.101997) reported on acetic acid production in the N2:CO2 fed bioelectrochemical system.

Thank you for this comment. We agree that other molecules can be obtained such as acetate. This particular point is now considered in the manuscript during the presentation of the more in-depth literature in accordance with the previous comment.

4. Lines 70-98: The generic introduction on microbial N2 fixation can be shortened considerably. Instead, the rationale and objectives of this study should be elaborated further.

Thank you for this recommendation. Part of the presentation of the N_2 fixation has been removed. Regarding the objectives, they have been revised as follows in Introduction section (l. 100–l. 110).

" In order to better understand the microbial mechanisms supporting N_2 fixation in polarized cathode enrichment and produce biomass, this work aims at developing an enrichment method of cathodic biofilms for direct fixation of CO_2 and N_2. Rago et al. (2019) demonstrated the capacity of producing biomass from air, CO_2 and a solid electrode polarized negatively (Rago et al., 2019). Here a specific enrichment of microorganisms capable of N_2 fixation was developed. It was hypothesized that a multi-step enrichment with specific medium could select a microbial community able to fix N_2 and CO_2 supported by electrons brought by a cathode. It was assumed that these communities enriched by this procedure could use a large number of interactions leading to N_2 fixation and biomass growth. For that, it was assumed that the enrichment in autotrophic diazotrophic bacteria in the presence of a cathode with pre-enrichment steps in presence of several electron donors (organic C and cathode) would be more efficient than enrichment cultures of hydrogenotrophic and diazotrophic bacteria."

5. Line 117-118: It is trace metal solution composition and not vitamin solution.

Thank you for pointing out this mistake. the composition of the vitamin and trace metal solutions have been revised as follows (l. 126-l. 130).

"The SL-6 trace element solution consisted of 0.1g $ZnSO_4$ $7H_2O$, 0.03g $MnCl_2$ $4H_2O$, 0.3g H_3BO_3 0.2 $CoCl_2$ $6H_2O$, 0.01g $CuCl_2$ $2H_2O$, 0.02g $NiCl_2$ $6H_2O$, and 0.03g Na_2MoO_4 $2H_2O$ per liter of solution. The vitamine solution consisted of 10mg Riboflavin, 50mg Thiamine-HCl $2H_2O$, 50mg Nicotinic acid, 50mg Pyridoxine-HCl, 50mg Ca-Pantothenate, 0.1mg Biotin, 0.2mg Folic acid and 1mg Vitamin B_{12} for 100 mL of distilled water."

6. Line 170: The reasons for applying these different steps in this particular enrichment approach should be stated.

In order to clarify the procedures, the objectives of each step have been added by small sentences in the material and method section related to the enrichment procedures (l. 196, l. 205, l. 211, l. 219).

"This first step was used to select N_2-fixing bacteria using organic compounds as electron donors.

Inoculation of the cathode in presence of organic C sources was made to favor bacterial growth.

Organic C sources were then removed and the cathode was used as sole electron donor in reactor. Here, only bacteria able to use cathode as electron source by direct interaction or indirect with H_2 were able to grow.

In the second enrichment method, the H_2-fed enrichment (H_2E), autotrophic bacteria were enriched in inorganic medium supplemented with H_2 as sole electron source."

7. Line 215-224 – estimation of nitrogen present in biomass –
    1. This method is not clear.

Thank you for the feedback on the calculation method. This point is addressed in the next answer to comments (comment 2).

2. For the estimation of actual bacterial count – what all genus were considered?

For bacterial count, the total abundance of bacterial *16S* rDNA as measured by qPCR together with the relative abundance of each OTU based on the sequencing of this *16S* rDNA gene were used to calculate the *16S* rDNA quantity per genus. Then the NCBI rrndb 5.7 database reporting the average amounts of *16S* genes per genome of many bacterial genera and families was used to estimate the "real" number of bacteria by dividing the measured *16S* gene amount per genus by the *16S* gene copies/bacterial genomes. When the genus was not present in the database, the average value of the related family was used.

The following sentence was added to clarify this aspect (l. 248- l. 251):

"Nitrogen present in the biomass (biofilm and planktonic) was estimated from quantification of 16S rDNA with qPCR. The rrnDB-5.7 database was used to estimate the actual bacterial amount from 16S rDNA qPCR using theoretical 16S rDNA copies/genome per strain, genus or family given by the database and sequencing results from our communities"

3. Why is theoretical data of E. coli used for estimations?

The mass of *E. coli* a model bacteria was used as bacterial mass constant in our calculations. In absence of a given mass for each of our strains, we chose to use this model for which the data was available. This mass is possibly different from the mass of the bacteria actually present in the enrichments. However, we assumed that this should not substantially impact the conclusions.

8. Line 243-244 –Why is nifH used as a marker and not any other nif gene? How is this specific N2 fixing activity calculated?

The *nifH* gene was selected as the only marker because it is the most widely used for monitoring and quantification of nitrogen-fixing bacteria (Dos Santos et al., 2012; Gaby & Buckley, 2012). Other markers such as *nifD* and *nifK* could also have been used. With regards to our objective of monitoring the increase of N_2-fixing bacteria, *nifH*, the gene coding for the Fe subunit common to all nitrogenases, was considered as sufficient.

Nonetheless, the following sentence was added in material and method section (l. 324-l. 326) :

"The nifH gene is known as marker of N_2 fixing bacteria, common to all nitrogenases and is used for their quantification because of its necessary presence for the fixation of N_2. (Dos Santos et al., 2012; Gaby & Buckley, 2012)."

About the specific activity, this one is measured using the number of bacteria theoretically capable of fixing N_2 measured by *nifH* qPCR and the amount of C_2H_4 produced. The quantity of *nifH* corresponds to a measurement made at the end of the batch unlike the measurement of C_2H_4 which was made during the batch but neglecting the possible variations of quantity of *nifH* during a batch. As with the estimation of N, changes in orders of magnitude were considered significant, ie. showing a minimum difference of one log between samples.

The next sentence was used to clarify the calculation (l. 289):

"This specific activity corresponds to the rate of C_2H_4 produced per bacteria capable of fix N_2 measured by qPCR of nifH gene."

9. The calculations for the acetylene reduction assay are missing in the methodology section.

Thank you for your comment on this missing point. Rates calculated from ARA measurement are based on the following formulas which have been added to the article in the methodology for gas subsection (l. 279–l. 287):

" eq7-> $n_{C2H4} = \frac{P_{NaOH} V_{C2H4}}{RT} = \frac{(P_{total} - P_{CO2}) \times V \times \%_{C2H4}}{RT}$

With (P_{total} the pressure in headspace, P_{CO2} the partial pressure of CO_2 measured without CO_2 trap, V the volume of gas in headspace, $\%_{C2H4}$ the part of C_2H_4 measured by GC-TCD in headspace, R the perfect gas constant and T, the temperature of the reactor.

And Acetylene production rate were calculated following next equation:

$$Eq8\text{->}\Delta[C_2H_4] = \frac{[C_2H_4]_{t1} - [C_2H_4]_{t0}}{t1 - t0} = \frac{\frac{n_{C2H4_{t1}}}{V} - \frac{n_{C2H4_{t0}}}{V}}{t1 - t0}$$

With $\Delta[C_2H_4]$ the rate of C_2H_4 production in µmol/L.d, t0 the last measure without C_2H_4 observed, t1 the first measure of C_2H_4 in headspace and V the volume of liquid which is constant between two ARA measurement."

10. Lines 345-350: The nifH gene and 16S rDNA gene copies were higher in the nPCE control than H2E experiment. What does it mean as far as the N2 fixation activity is concerned? Were there any other electron donors in the nPCE system?

The description of the enrichment procedures was not fully clear. Indeed, a first step of enrichment with cathodes was performed in presence of organic C. This organic C source could then be used as an electron source for the nPCE enrichment. Therefore, N_2 fixation could have occurred during this enrichment with organic C but was no longer possible, as shown by the results of the ARA method which confirms the absence of N_2 fixation for nPCE aft er340 days. Graphical representation of enrichment procedures, table and the following sentences have been added to the article to clarify this point.

The following statement has been added in the Results and discussion section (l. 397–l. 402)

"This higher biomass concentration was likely due to the enrichment period in presence of organic C (day 0 to 115 including 60 days in BES) for the PCE and nPCE enrichments. NH_4^+ in nPCE was also observed at a rate of 0.07 mg/L.d as presented in table 1 but without acetylene accumulation, meaning that no N_2 fixation occurred. In absence of electron source, nPCE enrichment communities could have been maintained through cryptic growth. The presence of NH_4^+ in the nPCE was likely related to cell lysis since no measurable fixation was detected by ARA even though significant biomass production was observed."

11. Lines 376-379: Any particular reason why there was no revival in the current density performance in spite of having the same experimental conditions after 260 days?

Some hypotheses could explain this observation, such as changes in the communities. Although we did not further discuss this part to avoid too much speculation, a sentence was added to illustrate one of our hypothesis (l. 573-l. 575).

"Therefore, autototrophic bacteria responsible for CO_2 fixation and heterotrophic bacteria that could also be H_2 dependent for N_2 fixation were greatly affected, leading to a decrease in the reduction reactions at the cathode and subsequently in current density."

12. Table 2: Though PCE 1 and PCE 2 are replicate reactors, a considerable difference in their performance during both periods (particularly the first period) was observed. Why so?

Thank you for this comment. Several explanations are possible starting with slight differences in communities leading to different reactions occurring in parallel. Exopolysaccharide produced by

biofilm can be a difference between both PCE cathodes. A difference in the rate of oxidation of the produced acetate due to slight difference in the community can also be an explanation.

These aspects are now more elaborated in the manuscript (l. 547-l. 550,l. 577–l. 581).

" The high coulombic efficiencies were probably associated to acetotrophic and acetogenic bacteria. Indeed, the acetate produced from electron collection at the cathode could have been used by acetotrophic bacteria for growth, lowering coulombic efficiency of acetogenesis and increasing CE for bacterial growth.

As seen in the table 3, electrons were retrieved in biomass production, N_2 fixation products, H_2 found in headspace and in CH_3COOH product from CO_2. These products were not sufficient to close the electron mass balance. The loss of electrons and the differences between cathodes of PCE 1 and PCE 2 was explained by side reactions, such as O_2 reduction or biological reaction such as exopolysaccharide (EPS) production."

13. Table 2 and CE discussion: What are the other possible electron sinks in the PCE reactors? A considerable loss in Coulombic efficiency is seen in both periods (very high in period 2).

As seen in previous comment, several electron sinks are possible to explain both the differences between the two reactors and incomplete electron balance. Indeed, aerobic conditions may be responsible for some of the electron capture. Biotic reactions, such as the production of EPS, are favored in one community over the other, where carbon is more strongly fixed. Moreover, de Fouchécour et al., 2022 have shown average CE of the order of 20% in a review on anodic BES. Which is of the order of magnitude of our results.

Changes were mentioned in the previous comment to clarify this point.

14. The microbial community discussion part is thorough and appreciated.

Lines 579-580 - How nifH/16s rDNA ratio are compared in terms of percentage? What does the percentage signify here? How can high nifH/16s rDNA ratio be speculated as presence of high N2 fixing bacteria?

Thank you for your comment. Indeed, it was not fully appropriate to estimate percentages for this ratio. Therefore, percentages have been removed to leave only the calculated ratio. The ratio was then used to monitor the evolution in time of the part of bacteria able to fix N_2. A high ratio indicated a high proportion of nitrogen fixing bacteria within the total community. As the number of copies of each gene is different from one bacterial species to another, changes of several logs was considered as significant.

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