Labor division of electroactive and carbon degrading microorganisms in bioelectrochemical laminar flow reactors

Yuting Guo[1,2], Luis F.M. Rosa[1], Yongping Shan[3], Falk Harnisch[1], Susann Müller[1]

1. Department of Environmental Microbiology, Helmholtz-Centre for Environmental Research – UFZ, Permoserstraße 15, 04318 Leipzig, Germany

2. Flow Cytometry Centre, National Institute of Biological Sciences – NIBS, Beijing, 102206, China

3. Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences – CAS, Beijing, 100012, China

ABSTRACT

The composition and functions of an anodic microbiome are key to both, successful wastewater treatment on the one and efficient current production on the other hand. How the microorganisms can effectively contribute to these different functions may depend on the type of substrate and the mode of cultivation. In laminar flow bioelectrochemical systems (BES) three different carbon sources with different degrees of complexity were studied, including acetate as benchmark substrate as well as defined wastewater (DW) and undefined wastewater (unDW) as further substrates. Off-line flow cytometry was used to perform a spatially-resolved analysis of the biofilms in the individual channels of the laminar flow BES. Along the nutrient gradients in the channels, biomass formation was heterogeneous with less biomass in downstream channels which was unfavorable for both, current production and carbon removal efficiency. The microbial compositions of reactors fed with unDW showed more similarities with reactors fed with acetate than with DW. Both, the cytometric α-diversity and intra-ß community diversity values followed a decreasing (in initial seven channels) and then increasing (downstream channels) overall trend, indicating a reduction in community complexity for all substrates and subsequently the restructuration of biofilm community. The division of labor between current production and substrate degradation was found to be independent of provided substrates, with some subcommunities performing dual functions as both electroactive microorganisms and carbon degraders. Yet, the degree and efficiency of division of labor, in terms of biomass formation as well as types of subcommunities, was depending on the substrates.

CORRESPONDING AUTHORS

Susann Müller: susann.mueller@ufz.de

Falk Harnisch: falk.harnisch@ufz.de

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INTRODUCTION

Biofilms are important in nature and relevant in a variety of technical processes, including wastewater treatment (Sgier et al., 2016), bioremediation (Singh et al., 2006; Syed et al., 2021), and the production of value-added products such as bioplastics and biofuels (Gunes, 2021; Khattab et al., 2021). Biofilms are also highly relevant for primary microbial electrochemical technologies, where they are formed at anodes or cathodes in bioelectrochemical systems (BES) (Schröder et al., 2015). The most prominent applications are microbial fuel cells (MFCs), microbial electrolysis cells (MECs), and microbial electrosynthesis (MES). Among the different applications treating wastewater (WW) is one key driver for advancement of BES and the entire field and certainly the best developed application scenario. The core element of all BES treating WW is the anode, at which the interplay of electrode material, architecture and microbial community of electroactive microorganisms (EAM) is the key for successful WW treatment and electron harvest (Kerzenmacher, 2017). Representative EAM are *Geobacter sulfurreducens*, *Geobacter metallireducens*, and *Shewanella oneidensis*. They are known to be able to exchange electrons directly with an electrode via membrane cytochromes or self-secreted mediators (e.g., *Shewanella*) (Koch and Harnisch, 2016). This interplay has been shown to be highly complex in numerous studies and always depends on the relationship between abiotic parameters such as substrate composition and concentration, reactor and electrode design, and the microbiome (Esquivel et al., 2020; Quejigo et al., 2021; Rago et al., 2021; Scarabotti et al., 2021). This complex interplay needs to be revealed in order to allow knowledge-driven engineering of BES (Conners et al., 2022; Noori et al., 2022).

Currently, the investigation on complex microbial composition mainly depends on sequencing technologies. High-throughput sequencing has been broadly applied to study the composition, function, evolution and interaction of microorganisms in various environments (Xu and Zhao, 2018). However, sequencing techniques are still far away from being routinely applied due to their complexity and need for elaborate data analysis, their time demand as well as high costs (Koch and Müller, 2018). Therefore, alternative high-throughput methods such as flow cytometry are coming into focus for microbial community analysis, which has been shown to provide quantitative and high-resolution assessment in near to real time and at lower cost than sequencing (Liu et al., 2019; Li et al., 2022). The application of flow cytometry for characterization and quantification of microbiomes in BES has been

introduced and is used increasingly (Harnisch et al., 2011; Koch et al., 2015; Pous et al., 2015; Esquivel et al., 2020; Guo et al., 2020).

In addition to the microbial community and anode materials (Kerzenmacher, 2017), the reactor architecture seems decisive for the formation of the "habitat" for the microbes and the shaping for niches (Logan and Rabaey, 2012; Krieg et al., 2019). We have recently introduced a tailor-made bioelectrochemical laminar flow reactor (Guo et al., 2020) being designed using finite-element modeling. In the bioelectrochemical laminar flow reactor that is also termed laminar flow BES within this article, concentration gradients of substrates can easily develop within the reactor channel length and height, resulting in different substrate concentrations and thus different accessibility for both electroactive microorganisms and non-electroactive microorganisms.

Consequently, the aim of this study was to discover the functionalities (e.g. carbon degradation and electric current production) of the microbial communities when using different substrates and the influence on microbial distributions (e.g. changes in composition and level of heterogeneity) in laminar flow BES. This shall gain a deeper understanding of microbial division of labor, especially EAM selection when facing complex matrices (e.g. refractory organics and readily fermentable organics) at different concentrations, providing insights in better BES engineering.

MATERIALS AND METHODS

Design of bioelectrochemical laminar flow reactors

The details of the design and assembly of laminar flow bioelectrochemical systems were described previously (Guo et al., 2020). In short, using the CAD interface of COMSOL Multiphysics (Comsol Multiphysics GmbH, Göttingen, Germany), the geometries were drawn, the resulting files were exported and sliced in the program Ultimaker CURA (Ultimaker, Geldermalsen, The Netherlands). The flow channel assemblies were printed using a 3D printer (Ultimaker 2+, Geldermalsen, The Netherlands) with the highest quality settings and 100% infill, using 2.75 mm Ø poly-lactic-acid (PLA) filament (Innofil PLA, BASF 3D Printing Solutions BV, Emmen, The Netherlands). Graphite plates (CP-2200®, CP Handels-GmbH Wachtberg, Germany) of 200 × 135 × 2 mm were used as electrodes. A reference electrode with 5 mm diameter and 100 mm length (Ag/AgCl sat. KCl, SE11, Xylem Analytics, Meinsberg, Germany) was placed inside channel 9 (SI Figure S1). A cation exchange membrane (FKE-50, Lot-No.: M21471301, FuMA-Tech, Ingbert, Germany) was sandwiched and glued (Loctite Hysol 3430, Henkel, Düsseldorf, Germany) between the anode and cathode assemblies. The final working volume of each anode and cathode chamber was 96 mL, in total 192 mL of one bioelectrochemical laminar flow reactor.

Experimental setup and operation

Two different fully water soluble substrate mixtures were studied. One substrate was defined wastewater (DW) (Riedl et al., 2017), using a mixture of six different carbon sources, including acetate (2.5 mM), glucose (0.4 mM), D-ribose (0.5 mM), glycine (3.3 mM), L-cysteine (2.0 mM), and potassium hydrogen phthalate (0.7 mM) with a total chemical oxygen demands (COD) of 842 mg L^{-1}. The second substrate, undefined wastewater (unDW), was composed of commercially available sweet whey powder (Süssmolke, Aurelia Nachbaur – Allgäuer Naturprodukte, Weiler-Simmerberg, Germany) as the only carbon source – mainly including 75% lactose, as well as fatty acids (0.7%), protein (12%) and salt (3.3%). The whey powder was added at a concentration of 800 mg L^{-1}, with COD of 838.4 mg L^{-1} (1 g whey powder was measured to contain ca. 1048 mg L^{-1} COD). The medium compositions of DW and unDW can be found in SI Table S1. A total of 11 reactors were built, of which 5 were used for the DW and 6 for the unDW substrates.

We have used the same reactor design and operating modes (batch followed by continuous feeding) as before (Guo et al., 2020) where the performance of EAM on 10 mM acetate as sole carbon source was studied. The results from this former study are considered and also used in this new study as benchmark (BM) results. In the BM study, the inoculated EAM grew as anodic biofilm dominated by *Geobacter* spp. (Harnisch et al., 2011) which were harvested from a potentiostatic controlled bioelectrochemical fed-batch reactor with reproducible maximum current density of 600 µA cm^{-2} (as described elsewhere, Gimkiewicz and Harnisch, 2013). This BM system was terminated after 35 d.

All experiments were operated at 35 °C using two modes of operation: an initial 7-day batch cultivation, followed by a continuous feeding with a mean hydraulic retention time (HRT) of 24 h. A schematic diagram of the experimental workflow is shown in SI Figure S2. The inoculum was a 1:1 mixture of i) enriched EAM and ii) wastewater collected after the primary clarifier of a wastewater treatment plant (WWTP) (Abwasserzweckverband (AZV), Borsdorf, Germany). The anode potential of the reactors was controlled using a multichannel potentiostat (MPG-2, Bio-Logic SAS, Claix, France), and kept at +200 mV vs. Ag/AgCl sat. KCl reference electrode. Details of inoculum preparation and reactor operation modes can be found in SI Section 1.

Sampling, sample measurement and data analysis

The COD (NANOCOLOR COD 1500, MACHEREY-NAGEL GmbH, Düren, Germany) and pH (LAQUAtwin pH Meter, HORIBA Europe GmbH, Oberursel, Germany) of the outlets of the reactors were measured every 1-3 days. The carbon sources present in the outlet of the DW systems were further quantified by HPLC (Agilent Technologies, Inc. CA, USA) as detailed in SI Section 2.

Current densities were calculated by dividing the recorded current by the anodic geometric surface area for each reactor (Harnisch and Freguia, 2012):

$$j = i \times S^{-1} \qquad \text{Eq. 1)}$$

where j is current density [A m^{-2}], i is current [A], and S is the anodic geometric surface area for each reactor [m^2], that is 0.017 m^2.

Coulombic efficiency (CE) of the system was measured considering the following equation:

$$CE = \frac{\int_0^t i \times dt}{\Delta COD \times \left(\frac{4}{32}\right) \times V \times F} \qquad \text{Eq. 2)}$$

where in numerator the i is integrated in the designated time between the initial and final measurement of COD, dt. On the denominator ΔCOD [g m^{-3}] is the variation (initial – final) of the COD, $\frac{4}{32}$ is the number (of moles equivalent) of electrons exchanged for every gram of oxygen equivalet oxidized, V is the treated wastewater volume [L] and F is the Faraday constant (96485 $\frac{A \cdot s}{mol}$).

The standard deviations of all chemical and electrochemical results shown below were calculated from five independent replicates of DW fed reactors, or six independent replicates of unDW fed reactors.

At the end of the continuous feeding, the laminar flow reactors were stopped for biofilm harvest: the anode chamber was carefully separated from the cathode, and the biofilm formed in each anodic channel was carefully scratched off into an individual microreaction vessel, and was resuspended in 2 mL of phosphate buffered saline (PBS, composition in SI Table S2). The biomass production in each channel was estimated via OD_{600} measurement. All samples per reactor (16 biofilm samples + 1 outlet sample) were then fixed using 2% paraformaldehyde solution and stored (in 70% ethanol) at -20 °C until off-line flow cytometric measurement. Details of the sample fixation and flow cytometric measurement can be found in SI Section 3. Cytometric data analysis and gate-template creation was done using FlowJo V10 (FlowJo, LLC, Oregon, USA). In total, the gate-template (SI Figure S3) included 31 gates, i.e., subcommunities that emerged in 190 samples. All 190 cytometric 2D-plots can be accessed at the FlowRepository (https://flowrepository.org/experiments/2790) with Repository ID FR-FCM-Z2N6. The flowCyBar tool (https://bioconductor.org/packages/release/bioc/html/flowCyBar.html) (Cichocki et al., 2020) was used to visualize the cytometric community dynamics by using gate information of all samples.

RESULTS

Bioelectrochemical reactor performance

Bioelectrochemical reactors fed with two types of substrates, respectively, were initially operated for seven days under batch conditions (SI Figure S4, in gray). During this time period, the current gradually increased and reached a maximum, indicating the possible formation of an EAM biofilm on the anodes. Then the current decreased due to the depletion of the carbon sources in the batch mode. Before the current reached zero, the continuous feeding mode was started.

During continuous operation, the feeding resulted first in a continuous increase of current density in the DW fed reactors (SI Figure S4A) to more than 10 µA cm^{-2} that subsequently averaged at 8.5 ± 1.7 µA cm^{-2}. The COD removal efficiency was 23.9 ± 9.6% and the coulombic efficiency (*CE*) was 61.0 ± 16.9%. The anode pH was stable over 30 d (7.9 ± 0.4, SI Figure S5). Interestingly, in the DW fed reactors, only glucose and D-ribose were consumed out of the six carbon sources (SI Figure S6). The enrichment of the fermentation products, e.g., acetate, resulted in an increase of their concentration at the outlet (3.1 ± 0.6 mM) compared to the inlet (2.5 mM). Thus, there was no full degradation of acetate that can serve as electron donor for several EAM into CO_2, biomass and electrons at an HRT of 24 h (Koch and Harnisch, 2016). Thus, the increased acetate concentrations at the outlet indicated that either the specific EAM were not sufficiently efficient to consume the overall acetate, or the EAM were only present in low abundance. We reason that the low cell numbers and heterogeneous distribution of biofilm in the channels may be the cause (SI Figures S7) of a lower conversion rate of the fermentation products to electricity.

During the continuous mode, the unDW fed reactors produced an average current density of 3.4 ± 1.4 µA cm^{-2}, the COD removal efficiency was 41.9 ± 24.0% and CE was 12.5 ± 15.0%. The low pH (5.4 ± 1.1, SI Figure S5) in the anode outlet hints for a higher rate of fermentation than the substrate-to-current conversion rate (hence the lower currents measured, SI Figure S4B).

Structure of reactor microbiomes

The microbial composition and spatial stratification of bioelectrochemical laminar flow reactors were studied by flow cytometry on the individual cell level in the biofilms for each reactor channel. The cells were sampled on the last day of the experiment after the reactors were opened. By flow cytometric analysis, cells with similar inherent information on light scattering (i.e., cell size related) and DNA content (i.e., chromosome number per cell related) were clustered as a subcommunity (i.e., gate, G). Whenever a new subcommunity became apparent, a new gate was set. The final gate-template with 31 subcommunities (SI Figure S3) was created and applied to each sample to determine the community structure variation within and between laminar flow BES on the basis of cell abundances per gate and sample (i.e., cell number per subcommunity, Koch *et al.*, 2014; Guo *et al.*, 2019).

Substrate complexity-dependent structure differences of microbial communities

The changes in cell abundance per subcommunity along the 16 channels as well as the outlet are shown as a barcode (SI Figure S8) for all laminar flow reactors (5 parallels for DW, 6 parallels for unDW). The data of the benchmark samples (Guo et al., 2020) were also included to compare the impact of different substrate complexity on the microbial community structures. The dissimilarities in community composition were visualized in dependence of the different substrate complexities in Figure 1 using Bray-Curtis measures (Koch et al., 2013). The compositions of the microbial communities were clearly different and dependent on the substrate feed. The composition of communities grown on acetate (BM) was more equal among channels and more similar to the communities grown on unDW. The structures of communities grown on DW were different from all other substrate conditions. In addition, communities grown on DW showed a unidirectional trend in the evolution of community composition (small dots to larger dots) in downstream flow of the reactors. Communities grown on the other two substrates (i.e. BM and unDW) did not show large differences in composition along the channels.

We also determined diversity values for the communities grown on the different substrates within channels 1-16 per reactor. Diversity metrics were determined using cytometric data on the basis of dominant subcommunities (threshold above average cell abundance of 2.95%, Liu *et al.*, 2019). The α-diversity values count the number of dominant subcommunities and showed an apparent decrease in community complexity in BM and a smaller decrease for the other substrates up to the channel number C6 (Figure 2A). This decrease in α-diversity suggest that compared to the original wastewater community only a few cell types were selected for the formation of the biofilm and the degradation of the carbon sources in the early channels. The intra-β-diversity values describe the differences in subcommunity numbers between successive channels and, therefore, changes in community composition between channels (Figure 2B). The intra-β-diversity between the early channels (C1-C3) followed a clear decreasing trend in BM and DW, pointing to the fact that communities were reaching balanced unchanging states in the subsequent channels (C3-C7). The intra-β-diversity changed little in the unDW channels, indicating that the community composition did not change much in these channels. Further downstream from C7, the intra-β-diversity in BM (e.g. C11-C14) fed reactors showed sudden increases. This trend was found less obvious in DW and unDW substrates in the mid channels (e.g. C9-C10). These variations point to larger community shifts. Interestingly, starting from C7, all feeding systems showed also increasing α-diversity values suggesting that more diverse cell types were involved in the restructuration processes and thus in the microbial electroactivity as well as the degradation of carbon sources. For the DW and unDW fed reactors, the intra-ß-diversity values decreased at C11, obviously establishing again a very stable community with a medium α-diversity.

The BM reactors was not as stable and showed high shifts in intra-β-diversity between the last channels of the laminar flow reactors.

Details of microbial diversity in terms of the presence and absence of dominant subcommunities in each channel of the three substrate systems can be found in Figure 3. For example, G2 was the subcommunity that was, independent of the used substrate, found in almost all channels of all reactors. On the contrary some subcommunities such as G21 occurred exclusively in DW and unDW, while G16 was found exclusively in BM. Some dominant subcommunities in BM disappeared below threshold such as G8, G12, G15, and G16 in DW and unDW. The loss and gain of particular subcommunities along channels per reactor depending on the substrate indicates diversification of functions between cell types in communities.

Division of labor within microbial communities with channel numbers

Microorganisms in the bioelectrochemical laminar flow reactors need to perform two major functions: i) oxidizing the substrates to remove carbon, and ii) shuttling electrons from the oxidation process to the anode, to create a current flow. The challenge for the microorganisms is to establish an effective ecological niche along the channels at the anode to promote both the COD removal and the current production by developing a well-balanced food web. Most complex substrates cannot be used directly by the EAM for growth and energy production (Koch and Harnisch, 2016). Therefore, a division of labor between fermentative microorganisms that break down complex molecules into suitable substrates for EAM is required. To visualize this division of labor, i.e. “who” is doing “what” in sequence, a possible microbial stratification along the laminar flow channels was studied by flow cytometry.

Microbial community functions along the 16 channels were suggested by correlation analysis using Spearman correlation coefficient rho on the variation of cell number in all gates of the gate template and the formation of biomass associated with each of the channels (Figure 4). Cytometrically determined dominant subcommunities (cell abundance > 2.95%) that showed a significant positive correlation ($p<0.2$, $roh>0.5$) between cell number increase and biomass formation per channel were marked with yellow stars. Biomass formation depends on carbon source uptake and metabolism and is thus connected to carbon removal. Subcommunities with increasing cell abundances that were positively correlated with biomass increase were therefore considered as carbon degraders. Different types of degraders, i.e., different subcommunities, were found reactors fed with all three substrates. In BM there were 5 dominant degrader subcommunities in gates G2, G8, G12, G15 and G16 (Figure 4). In BES treating unDW, even more dominant subcommunities were positively involved, G2-G4, G6, G9, G13, G14, and G25, which were of relatively lower DNA contents and smaller cell sizes than those in

BM. When using DW only two subcommunities, G1 and G11, significantly served as carbon degraders. The position distribution of the degraders in the gate template can be found in SI Figure S3 (grey gates).

It was obvious that electron harvest, i.e. current production, occurred during carbon degradation, i.e. carbon resource utilization. Gates G2, G8, G12, G15-G17 and G20 had been reported to be the EAM *Geobacter* spp. (Guo et al., 2020). In the BM (fed acetate and terminated after 35 d), similar EAM-like subcommunities G2, G4, G8, G12, G14-G16, and G25 were the dominant cell types in most channels (Figure 3), and of these, the highest cell concentrations (e.g. up to 6×10^{11} cells mL^{-1} in channel 2) were found in the first seven channels. It is more than likely that these gates are involved in current production. Correspondingly, BM had the highest total charge production of 1.7×10^4 C compared to the other two substrates (Figure 5). Only for the BM the EAM-associated gates G2, G8, G12, G15 and G16 were also significantly positively correlated with carbon removal (Figure 4) indicating that for the BM the same cells were both carbon degraders and current producers. In contrast, in DW and unDW fed reactors, cell concentrations of EAM (*Geobacter* spp. associated gates) in all channels decreased significantly to 1.9% and 13.9% of BM values, and power production decreased to 23.8% and 6.9% of BM values, respectively (Figure 5, SI Table S3). In the unDW fed BES, biomass decreased within the first 10 channels and the dominant EAM-likely gates only showed up in G2 and G8 (Figure 3), with G2 being the most abundant. The other dominant subcommunities were G1, G3-G4, G9, G14, and G25 most of which showed a positive correlation to COD removal (Figure 4). Therefore, it can be assumed that in the unDW fed reactors the majority of dominant subcommunities was involved in carbon degradation and not in current production. All of them were equally distributed between all channels. In the DW fed reactors, showing the lowest biomass formation, the biomass decreased already after 2 channels (SI Figure S7). Only G2 showed a dominant presence of the EAM found in the earlier study and was, as in BM and unDW, the most abundant subcommunity. Of the other dominant subcommunities G1, G4, G9, G11, G14, G21 and G25, only G1 and G11 showed a significant positive correlation to biomass production. Therefore, it can be assumed that the subcommunity in G2 was a highly active subcommunity in current production, while fewer subcommunities compared to unDW were involved in carbon removal. As before in unDW, these subcommunities were evenly distributed along all channels.

The data clearly show that some subcommunities perform dual functions as both EAM and carbon degraders, such as gates G2, G8, G12, G15, and G16. The remaining dominant subcommunities G1, G3-G4, G6, G9, G11, G14 and G25 contained those microorganisms that were significantly positively correlated with biomass production and carbon removal (Figure 4). Therefore, we can assume that there is a division of labor between them along all channels and regardless of the substrate used in the

bioelectrochemical laminar flow reactors. At the same time depending on the substrate provided the efficiency with respect to the two functions was quite different.

DISCUSSION

When considering the entire geometric surface area of the anode, the current density of the bioelectrochemical laminar flow reactors was relatively low with average values of 8.5 ± 1.7 µA cm^{-2} and 3.4 ± 1.4 µA cm^{-2} when fed with DW and unDW, respectively. Other studies have also been using whey powder as a carbon source for unDW, but with different setups and media compositions as well as use of inhibitors (such as 2-bromoethanesulfonate to prevent methanogenesis) and different anode surface-to-volume ratios, anode materials and mass transfer regimes (Antonopoulou et al., 2008; Patil et al., 2011; Blanchet et al., 2015; Wenzel et al., 2017; Esquivel et al., 2020). They showed a wide range of *j* ranging from 3 µA cm^{-2} to 310 µA cm^{-2}. Generally, the anode surface-to-volume ratio (anode/medium) was highest in our laminar flow BES with 1770 cm^2 L^{-1}. Comparable to the results using DW as substrate Riedl et al. (2107) showed a 21 times higher current density but with a 63.2 times lower surface-area-to-volume ratio (28 cm^2 L^{-1}). In addition, the biofilm was more uniformly distributed on the anodes operated in stirred tank reactors, whereas in the laminar flow BES used here, a heterogeneous biomass distribution was intended due to the formed substrate gradients. Whether such heterogeneous biomass formation resulted in a stratified microbial composition leading to a partitioning of functions between cell types is discussed below.

It has been reported that EAM are metabolically limited due to the fact that they utilize preferably small organic acids (Kiely et al., 2011; Koch and Harnisch, 2016). Therefore, the degradation of complex carbon substrates has to be performed by other, probably non-electroactive members of the community (Koch et al., 2019) in DW and unDW fed reactors. Different types of microorganism comprising different metabolic capacities are needed to ferment these components to short chain carbon sources being suitable for EAM. In the unDW fed reactors, the lactose degradation requires a conversion into galactose and glucose, followed by homolactic fermentation via the glycolytic pathway, while the pentoses (D-ribose) in the DW systems are fermented through the heterolactic phosphoketolase pathway to lactate and acetate (McLeod et al., 2010). The distinct metabolism of the different carbon sources seems to have been associated with different cell types and thus community structures and also with different efficiencies in carbon removal and biomass formation.

Interestingly, the α-diversity of the BM reactors decreased in the first channels which points to strong niche sorting forces for the EAM accompanied by a decrease in intra-ß-community diversity, indicating the establishment of the community structures. The same process was not as obvious for both DW and unDW fed reactors suggesting that no strong forces on the EAM by formation of niches were in place.

Furthermore, all three types of substrates fed reactors showed huge increases intra-ß-diversity values in the later channels which points to restructuration processes in the communities and a possible displacement of EAM. This process led to even higher α-diversities of all communities although the overall biomass values were quite low (Figure 2 and SI Figure S7). The displacement of EAM by insignificant fractions of cells that are probably serving only as carbon degraders might be assigned to the gradual depletion of substrates or due to the flow rate tailored better to degrader growth requirements than EAM. Accumulated acetate was found in the outlet of DW fed reactors pointing to the fact that the EAM were not or less active anymore after complex substrate degradation. In the unDW the lowered pH points to the degradation of the complex substrates but as before, the low CE points to low activity of EAM.

It is worth noting that when having a laminar flow BES of 3.2 m length in total and 24 h HRT, the biomass in all bioelectrochemical laminar flow reactors was clearly below 1×10^{11} cells mL^{-1} after channel seven due to the nutrient gradients. In general, BM fed reactors had the highest biomass, while it decreased to 16.9% in DW fed and 27.8% in unDW fed BES (sum of cells in dominant gates, Figure 5). For using complex DW and unDW, a longer HRT than 24 h could be proposed to achieve further degradation of nutrients and better current production. In addition, the metabolic pathways for bioeletrochemical transformation of the tested organic substrates could be studied in follow-up work for elucidating in-depth the microbial distribution and functionality restructuration. Further, an optimized assembly of the inoculum could be considered in advance to improve the performance of the bioelectrochemical laminar flow reactors, especially to improve the COD removal and CE compared to this study.

CONCLUSION

In our study we found a division of labor between current production and COD removal. No stratification between EAM and carbon degraders was found and the dominant subcommunities were stably established in the biofilm. Further downstream in the bioelectrochemical laminar flow reactors, biomass steadily decreased. In the BM reactors the EAM built their niche in the first channels which was not as obvious in the DW and unDW fed reactors (Figure 3). Nevertheless, community structures changed in the later channels due to higher diversity by obviously less dominant subcommunities (Figures 2, 3). EAM seem to be displaced by the carbon degraders and presence of organisms that are able to use specific pathways such as the pentose metabolisms seem to be ecologically at a disadvantage. Therefore, it could be projected that suitable combinations of channel numbers or appropriate HRTs are needed before dealing with different complexities of substrates. A fitting design would promote the reactor microbiome to efficiently carry out its duties for making full use of nutrients

resulting in a high current production as well as an treatment, e.g., longer HRTs in case of refractory organics (e.g., DW and unDW) and shorter HRTs in case of readily fermentable organics.

AUTHOR CONTRIBUTION

YG designed and conducted the experiments, evaluated the data and wrote the paper. LR designed the reactors, contributed to the experiments and writing. YS contributed to the data analysis and writing. FH designed the experiments and contributed to the data analysis and writing. SM contributed to the data analysis and writing.

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FIGURES

Figure 1. Similarity distribution of microbial communities in three types of bioelectrochemical laminar flow reactors. Benchmark (BM, in blue), defined wastewater (DW, in grey with 5 color gradients corresponding to 5 reactors) and undefined wastewater (unDW, in pink with 6 color gradients corresponding to 6 reactors) fed reactors are shown. Each channel is shown as a dot and the dot size increases with increasing channel number from 1 to 16. Open dots are outlet samples. Wastewater sample is in brown color, *Geobacter* spp. is in red color, and the inoculum is in orange and brown color, which was a mixture of enriched electroactive microorganisms and wastewater.

Figure 2. The α-diversity and intra-β-diversity values of three types of bioelectrochemical laminar flow reactors. (A) The α-diversity values along the channels of the bioelectrochemical laminar flow reactors: benchmark (BM, blue line, 3 parallels), defined wastewater (DW, grey line, 5 parallels) and undefined wastewater (unDW, red line, 6 parallels) fed reactors. (B) The intra-β-diversity values along the channels by comparing between each two successive channels, e.g. C2 with C1 (2-1), C3 with C2 (3-2) and so on. For diversity determination, only dominant gates above threshold of 2.95 % were taken into account.

Figure 3. Barcoding of the microbial communities in the bioelectrochemical laminar flow reactors. The bar codes represent the dominant gates (cell abundance above threshold of 2.95 %, marked in reddish) per channel (C1 to C16) in parallel reactors in benchmark (BM, the 3rd parallel), defined wastewater (DW, 5 parallels) and undefined wastewater (unDW, 6 parallels) fed reactors. The more cells, the more intense the red color. Gates with cell abundance below the threshold (2.95 %) were marked in blue. The curves in the figures show the cell number concentration of the dominant gates per channel, where the red lines were for *Geobacter* spp**.** associated gates 2, 8, 12, 15, 16, 17 and 20 and blue lines were for biomass formation associated gates: 2, 8, 12, 15, 16 for BM; 1 and 11 for DW; 2, 3, 4, 6, 9, 13, 14, 25 for unDW fed reactors (gate-setting details are shown in Figure 4).

Figure 4. Correlation analysis between cell abundance per gate and biomass per channel of bioelectrochemical laminar flow reactors (R). The data are from 3 benchmark (R1-R3 in BM), 5 defined wastewater (R1-R5 in DW), and 6 undefined wastewater (R1-R6 in unDW) fed reactors. Dominant gates (cell abundance above the threshold of 2.95 %) that show significant positive correlations ($p<0.2$, $roh>0.5$) with biomass production are marked with yellow stars. The details of the color key histograms of each reactor system can be found in SI Figure S9.

Figure 5: The respective cell concentrations and current production of BM, DW and unDW fed reactors. The cell concentration was the sum up of cells in dominant gates (threshold above average cell abundance of 2.95%), in gates connected to carbon degradation and in gates connected to current production in 16 channels. Current producing gates are *Geobacter spp.* associated gates: G2, G8, G12, G15-G17 and G20 for all three substrate fed reactors, and carbon degrading gates are assigned according to Figure 4: gates 2, 8, 12, 15,16 for BM, 1 and 11 for DW, 2, 3, 4, 6, 9, 13, 14, 25 for unDW fed reactors.