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Abstract

This study evaluated the impact of inoculum source and anode surface modification (carboxylate -COO^- and sulphonamide -SO_2NH_2 groups) on the microbial composition of anode-respiring biofilms. These two factors have not previously been considered in detail. Three different inoculum sources were investigated, a dry aerobic soil, brackish estuarine mud and freshwater sediment. The biofilms were selected using a poised anode (-0.36 V vs Ag/AgCl) and acetate as the electron donor in a three-electrode configuration microbial fuel cell (MFC). Population profiling and cloning showed that

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all biofilms selected were dominated by *Geobacter* sp., although their electrochemical properties varied depending on the source inoculum and electrode surface modification. These findings suggest that *Geobacter* sp. are widespread in soils, even those that do not provide a continuously anaerobic environment, and are better at growing in the MFC conditions than other bacteria.

Keywords: *microbial fuel cell; electroactive biofilm; soils; electrode surface; population profiling.*

1. Introduction

Exoelectrogens are found in anaerobic sediments and soils where they have access to both reduced organic compounds, for use as electron donors, and insoluble inorganic electron acceptors including manganese and iron oxides (Lovley, 1993; Weber et al., 2006). Many locations meet these requirements while varying in other environmental parameters. Previous work has confirmed the presence of exoelectrogenic bacteria in various different environments including freshwater sediments (Chae et al., 2009; Holmes et al., 2004), marine sediments (Bond et al., 2002; Tender et al., 2002), salt-marshes (Holmes et al., 2004), anaerobic sludge from potato processing (Rabaey et al., 2004), wastewater treatment plants (Kan et al., 2011; Lefebvre et al., 2010), and recently in mangrove swamp sediments (Salvin et al., 2012). *Geobacteraceae* are usually the predominant microorganisms colonizing the anodes introduced in such environments, with a higher abundance of *Desulfuromonas* species in marine and salt-marsh sediments; while in freshwater sediments, *Geobacter* species are the most common *Geobacteraceae* (Holmes et al., 2004). Following the Baas-Becking

hypothesis (1934) that "*Everything is everywhere, but the environment selects*", we should expect to select for exoelectrogenic biofilms dominated by *Geobacteraceae* whatever the inoculum used. Indeed, Yates et al. (2012) showed that the predominance of *Geobacter* sp. in acetate-fed MFCs (Microbial Fuel Cells) was independent of the inoculum source, after testing three inocula (two wastewaters from different locations and an anaerobic bog sediment). However, other researchers found that the inoculum makes a difference in the selection of anode-respiring biofilm in MFCs (Miceli et al., 2012). Miceli et al. (2012) tested thirteen samples from locations around the world and placed them in MFCs with electrodes poised at -0.30 V vs Ag/AgCl in acetate medium. Only 7 out of 13 samples produced sufficient current (>1.59 A/m^2) after 21 days of selection. They found that bacteria related to the genus *Geobacter* dominated only two of the seven biofilm communities producing a high current; the other biofilm communities contained different known and/or novel exoelectrogenic bacteria (Miceli et al., 2012). Few studies have looked at the effect of inoculum source on the composition of exoelectrogenic biofilms selected in MFCs either with or without fixed anode potentials. To bring more consistency in the results, it is recommended to test inocula in MFCs held at the same fixed potential (e.g., -0.08 V vs SHE), as the anode potential is likely to influence the composition of the anodic biofilm (Commault et al., 2013). The inocula tested in previous studies are typically from rich, moist anaerobic environments likely to contain *Geobacter* sp. In this study three very different inocula are tested: a saline estuary mud; a freshwater sediment; and a dry, exposed, low fertility basalt/loess soil thought to be unlikely to contain *Geobacter* sp. Each inoculum was placed in an MFC with the anode held at -0.36 V vs Ag/AgCl (-0.08 V vs SHE) as an electron acceptor and provided acetate as an electron donor. The selected anodic biofilms were

compared for current production, biofilm/electrode interaction, and dominant microbial community composition.

We also investigated the impact of electrode surface properties on the selection of electro-active biofilms in MFCs. The anode surface chemical and physical properties affect bacterial adhesion and electron transfer process between bacteria and electrodes (Guo et al., 2013). Modification of electrode surfaces aiming to improve the efficiency of MFCs has recently emerged as a new field of research (Kumar et al., 2013; Wei et al., 2011). Although some studies have proven that certain anode modifications lead to more efficient MFCs (Lapinsonniere et al., 2013; Picot et al., 2011), the influence of surface modifications for biofilm growth and maintenance is not well understood. In this study, the effect of two different chemical groups: negatively charged carboxylate group ($-COO^-$) and sulfonamide group ($-SO_2NH_2$) neutral at physiological pH were tested on electro-active biofilms selected in MFCs using the same inoculum and same anode potential (-0.36 V vs Ag/AgCl). The sulfanilamides are characterized by their lipophilicity and their amine groups partly protonated at pH 7. Note however that the amine group is lost in the modification process so that the resulting modifier bears a neutral charge (phenylsulfonamide). The lipophilicity of sulfanilamides favours their interactions with the lipid bilayer of the bacterial cell membrane and the polymeric lipophilic compounds of EPS (extracellular polymeric substances). The presence of phenylsulfonamide at the electrode surface is therefore likely to encourage the attachment of bacteria via lipophilic attachment. The carboxylates ($-COO^-$) are negatively charged at pH 7 (pKa(-COOH/$-COO^-$) ~ 4), which could potentially repulse bacteria. The bacterial community composition of biofilms selected on modified electrodes was investigated along with their electrochemical properties.

This paper examines whether two independent factors, inoculum source and electrode surface modification, could alter the composition and electrochemical properties of anodic biofilms selected in MFCs. This question is of importance for the discovery of new anode-respiring bacteria and new metabolic pathways for higher current production in MFC. The two factors were tested independently starting with three different microbial inoculum sources.

2. Methods

2.1. Electrode modification procedures

Carboxylate and sulfonamide groups were grafted onto graphite rod electrodes using the electrochemical reduction of aryl diazonium salts, as described by Picot et al. (2011). The process involved two steps, the formation of aryl diazonium salts from their corresponding amines followed by *in-situ* electro-reduction of the diazonium, by cyclic voltammetry with monitoring of the charge consumed in the process to control the amount of molecules grafted on the electrode (Picot et al., 2011). Diazonium salts were generated *in situ* in a total volume of 75 mL of acidic aqueous medium (0.1 M HCl) containing the starting aryl amine (4 mM of 4-aminobenzoic acid for $-COO^-$ and 2 mM of 4-aminobenzenesulfonamide for $-SO_2NH_2$) and sparged with argon for 10 min to remove oxygen. Then sodium nitrite ($NaNO_2$) was added at a final concentration of 10 mM. The mix was kept on ice in the dark to stabilize the generated aryl diazonium salt. This solution served as the electrolyte for the modification of the previously sandpapered graphite electrode by electrochemical reduction of the diazonium salts using a potentiostat (model EA164 QuadStat). A three-electrode cell configuration was used with an Ag/AgCl, NaCl (3 M) reference electrode (0.28 V vs SHE, BASI

Electroanalytical Chemistry, MF-2052) and a second graphite electrode as the counter electrode, as described by Commault et al. (2013). Electrochemical reduction of the diazonium salts was achieved by recurrent cyclic voltammetry sweeps starting at zero-current potential (around +0.2V vs Ag/AgCl) and decreasing to -0.2 V vs Ag/AgCl. Several scans at a rate of 0.05 $V.s^{-1}$ were needed to reach a global charge density (Q) of 15 - 20 $mC.cm^{-2}$ (projected anode area of 5.81 cm^2). To probe the effect of the modification on the electrode properties, cyclic voltammetry was performed at a scan range of -0.1 V to 0.4 V and a scan rate of 0.1 $V.s^{-1}$ in a solution of potassium ferricyanide $K_3[Fe(CN)_6]$: 2 mM of ferricyanide, 0.1 M KCl and 10 mM of phosphate buffer pH 7. The voltammograms obtained were compared to an unmodified graphite electrode.

2.2. Anode-respiring biofilm growth and selection

All the anode-respiring biofilms presented in this paper were selected in 100 mL MFCs as previously described by Commault et al. (2013). The anode potentials were maintained at -0.36 V versus Ag/AgCl (i.e. -0.08 V vs SHE) using a three-electrode arrangement. The counter electrode (carbon cloth, Fuel Cell Earth LLC, Ma, USA) was separated from the anolyte by an Ultrex CMI-7000 cation-exchange membrane (Membranes International Inc., NJ, USA) in a chamber containing 0.1 M phosphate buffer (pH 7.5). The anode, a (modified or unmodified) graphite rod of 5.81 cm^2, was maintained at a fixed potential by a 4-channel potentiostat (model EA164 QuadStat) connected to an e-corder 1621 unit (eDAQ Pty Ltd, NSW, AUS). The same inoculum was used for the experiment comparing the effects of two chemical groups grafted on anodes. The COO^- and SO_2NH_2 MFCs were both inoculated with 50 mL of water-saturated soil collected in Lincoln (Christchurch, NZ). For the experiment comparing

the effect of three different inocula on the growth and selection of anodic biofilms, 50 mL of soils from diverse environments were added to three different MFCs with unmodified working electrodes. The inocula were referred to as (i) "Crater Rim" (CR) a dry soil collected on the hillside of a Banks Peninsula walking track (Canterbury, NZ); (ii) "Church Bay" (CB) a wet saline estuary mud (Canterbury, NZ); and (iii) "Halswell River" (R) a wet soil from the bed of a freshwater stream (Canterbury, NZ). Once inoculate, the 100 mL MFCs were filled with a minimal medium (pH 7.5) containing 15 mM of acetate (composition described in Commault et al. (2013)) previously sparged with nitrogen (<10 ppm of O_2) gas for 10 min. The biofilms were left to develop on the constant-voltage anodes for 29 days (66 days for "Crater Rim") at room temperature (21°C) without mixing. The biofilms were fed 50 mL of fresh, nitrogen-sparged, acetate medium (15 mM acetate, pH 7.5) every two or three days in batch mode, corresponding to hydraulic retention times of 96 hours (4 days) and 145 hours (6 days) respectively. Current measurements were made every 10 min to follow the formation of anode-respiring biofilms. The experiment comparing the different inocula was not replicated due to the limited number of channels of the potentiostat.

2.3. Electrochemical analysis

Prior to each electrochemical analysis, the totality of the used medium (100 mL) was replaced in each anode chamber with fresh acetate medium (15 mM) to ensure that the pH and the chemical oxygen demand were the same for all the MFCs. Power density curves were plotted 29 days after selection under acetate saturation using the potentiostat (model EA164 QuadStat) and a two-electrode cell configuration by coupling the reference electrode with the counter electrode and poising the anode versus the counter/reference electrode, as described by Picot *et al.* (2011). Ten different

voltages were applied for 300 s from open circuit potential to near short-circuit potential, while monitoring the steady state current. The internal resistance of system (R_{int}) is obtained at maximum power and was calculated using equation (1).

$R_{int} = \frac{P_{max}}{I^2}$ (1), with P_{max} the maximum power and I the corresponding current.

The electronic interactions at the interface of the biofilm/electrode were measured by cyclic voltammetry 29 days after selection (66 days for "Crater Rim"). Cyclic voltammetry was performed using a potentiostat (EC epsilon, BASi, IN, USA) at 1 $mV.s^{-1}$ in turnover conditions and at 25 $mV.s^{-1}$ in non-turnover conditions at potentials ranging from -0.6 V to 0.1 V vs Ag/AgCl. The cyclic voltammogram of the electrolyte from the "Crater Rim" MFC was performed using a clean graphite rod electrode of 5.81 cm^2 and the same parameters as in non-turnover conditions, after 10 min of bubbling with nitrogen (<10 ppm of O_2) gas.

2.4. DNA extraction

DNA was extracted at the end of the experiment using an UltraClean™ Soil DNA Isolation kit (MO Bio Laboratories Inc., CA, USA). The extracted DNA was quantified by spectrophotometry (NanoDrop® ND-1000) and its quality examined by electrophoresis on 0.7% agarose gel. The DNA extracts were then stored at -20°C for further analyses. We used ARISA (automated ribosomal intergenic spacer analysis) for our analysis of bacterial communities because of the relative ease, cost-effectiveness and reproducibility of the method. ARISA is a fast method to visualize the taxon richness of a biofilm. In the case of biofilms with low taxon richness, cloning was performed to identify the species dominating the biofilms.

2.5. Population profiling: Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA is a semi-quantitative molecular DNA fingerprinting technique targeting the intergenic region of bacterial 16S and 23S rRNA genes. As length of the intergenic region varies across taxa, these data can provide a profile of community structure within each sample. ARISA of bacterial DNA was performed as previously described by Commault et al. (2013). The results were visualised in GeneMapper software (version 3.7, Applied Biosystems Ltd.) and processed in Excel. The similarities between the bacterial community data among samples were compared using a Bray Curtis similarity matrix (Legendre & Legendre, 1998) and visualised in the form of a cluster dendogram in PRIMER6 software (version 6.1.12, Primer-E Ltd., Plymouth, UK) (Commault et al., 2013; Lear et al., 2008).

2.6. Cloning and sequencing

The 16S rRNA genes of the extracted DNA were amplified using the universal primers B342If (5'-CTA CGG GIG GCI GCA GT-3') and U806Ir (5'-GGA CTA CCI GGG TIT CTA A-3') (Hori et al., 2006), except for the "Crater Rim" sample where the 16S rRNA genes were amplified using the universal primers PB36 (5'-AGR GTT TGA TCM TGGCTC AG-3') and PB38 (5'-GKT ACC TTG TTA CGA CTT-3') (Lear et al., 2009). The BIOTAQ™ PCR kit (Bioline), and the following PCR conditions were used: (i) 94°C for 3 min; (ii) 30 cycles of 94°C for 60 s, 50°C for 60 s, 72°C for 70 s, and then (iii) 72°C for 10 min. Once amplified, the 16S rRNA fragments were ligated into pCR2.1 vectors using a TA Cloning® Kit (Invitrogen, USA) and transformed into One Shot® *E. coli* TOP10F' $CaCl_2$ competent cells, according to the manufacturer's protocol (Invitrogen, USA). Isolated clones were selected on Luria Bertani (LB) agar plates

containing 100 µg.mL^{-1} of ampicillin. The inserts of clones were sequenced on a 3130XL Capillary Genetic Analyser (Applied Biosystems Ltd., Melbourne, Australia) in both reverse and forward directions using the plasmid specific primers M13f (5'-CTG GCC GTC GTT TTA-3') and M13r (5'-CAG GAA ACA GCT ATG AC-3'). The forward and reverse sequences were aligned and corrected with ChromasPro software (Technelysium Pty Ltd, Brisbane, Australia). The consensus sequences were compared with the nucleotide collection (nr/nt) of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/) using the megablast algorithm to confirm the likely identity of bacteria based on 16S rRNA gene sequence fragments.

2.7. Statistical analysis

Welch's t-tests were calculated using the excel function 'T.TEST'. Two-tailed t-tests were performed assuming unequal variance. At $p < 0.05$, we interpreted the data as being significantly different.

3. Results and Discussion

3.1. Effect of different inocula on the selection of *Geobacter*-dominated biofilms

Three MFCs were set-up with unmodified electrodes and inoculated with three different soil samples. No current was observed immediately after inoculation (Figure 1) allowing time for the bacterial community to adapt to the MFC conditions. A positive (oxidation) current was first observed in the MFCs inoculated with "Church Bay" (CB) and "River" (R) soils approximately 14 days after inoculation, followed by "Crater Rim" (CR) MFC after 28 days. The "Church Bay" soil was collected in an estuary where the salinity was likely higher than the salinity of the growth medium, but it did not seem to impact the start-up time of the MFC compared to the freshwater inoculum.

The start-up time of "Crater Rim" MFC was twice as long as the start-up times of the MFCs inoculated with "River" or "Church Bay" soils. When Miceli et al. (2012) tried to select anode-respiring bacteria from thirteen diverse inocula around the world using a poised anode (−0.30 V vs Ag/AgCl) and acetate as the electron donor in a MFC, only half of them produced high current densities (>1.5 A/m^2, anode) after 21 days of selection. They explained that their results might be due to factors such as the suitability of the media for the growth of the organisms present in the inocula, varying capacities for electrode respiration, or differing methods of biofilm formation between different organisms (Miceli et al., 2012). These factors could explain the longer start-up time of the "Crater Rim" communities too. The soil collected from the hillside of "Crater Rim" walking track was very dry compared to "Church Bay" and "River" soils, and was likely to contain mainly aerobic bacteria. Three potential reasons could explain the longer start up times seen with the "Crater Rim" inoculum: (i) there may be fewer bacteria capable of anode-respiration present in the inoculum, so it takes longer to multiply up to the numbers needed to produce a measurable current; (ii) the inoculum may contain less diversity amongst the anode-respiring bacteria than is present in the other inocula and therefore may lack species or strains well adapted for growing in the MFC conditions; (iii) "Crater Rim" bacteria may not have been exposed to anaerobic conditions for some time, and so it takes them longer to physiologically adapt to the anaerobic conditions or to form appropriate syntrophic associations that allow them to effectively colonize the electrode (physiological adaption). The results presented here, do not exclude any of these possibilities. It is plausible that the slow start-up of "Crater Rim" MFCs was due to a combination of all three reasons.

The current delivered by "Church Bay" and "River" MFCs was higher than 1.7 $A.m^{-2}$ towards the end of the experiment, while the maximal current density generated by "Crater Rim" MFC was only 0.13 $A.m^{-2}$ after 60 days of growth. Surprisingly, the current density doubled to reach 0.25 $A.m^{-2}$ when the biofilm was fed minimal medium without acetate after 66 days of operation (Figure 1). This is paradoxical as acetate was the only electron donor in the medium. It is possible that the high acetate concentration had an inhibitory effect on the electron transfer of the "Crater Rim" biofilm. Feeding the biofilm with acetate-depleted medium would have diluted the acetate left in the electrolyte to an optimal concentration for the biofilm. To have a better understanding of the mechanism of electron transfer in the "Crater Rim" biofilm, cyclic voltammetry and sequencing results were then considered.

The turnover voltammograms of "Church Bay" and "River" biofilms, performed after 29 days of enrichment, showed a sigmoidal catalytic wave characteristic of acetate oxidation by *Geobacter* sp. at the potential of outer membrane cytochromes with a midpoint potential around -0.4 V vs Ag/AgCl (Figure 2a,b). The "Church bay" and "River" voltammograms showed a complex pattern (Figure 2a,b), suggesting that two different pathways were involved in the electron transfer. At low potentials (between -0.4 to -0.3 V vs Ag/AgCl) the electron transfer was favoured by one pathway, and was then shifted to another pathway at potentials higher than -0.3 V. This dynamic potential-dependent change between two electron transport pathways was recently described by Yoho et al. (2014) in anode biofilm of *Geobacter sulfurreducens*. The "Crater Rim" biofilm had a distinct turnover voltammogram revealing the presence of a redox system with a reduction peak at -0.35 V and an oxidation peak at -0.05 V vs Ag/AgCl ($E_{1/2}$ = -0.15 V). The same redox system was observed on non-turnover voltammograms, as the

biofilm kept on producing the mediator even in the absence of acetate. This redox system was not detected in the electrolyte (Figure 2c) and can be assigned to either a redox active species produced by the "Crater Rim" biofilm or an outer membrane redox protein. In any case, this redox system does not seem to be involved in efficient direct or mediated electron transfer between the biofilm and the anode.

The dominant 16S rRNA genes of the three MFC biofilms were sequenced at the end of the experiment to confirm the presence of *Geobacter* sp. within their bacterial communities. Of 12 clones isolated from "Church Bay" biofilm, 11 were most similar to *Geobacter psychrophilus* with 96% of identity (Figure 3). The same *Geobacter* species dominated the "River" biofilm as 8 clones out of 9 were similar at 96% to *Geobacter psychrophilus* (Figure 3). When aligned together, the 16S rRNA fragments of "Church Bay" and "River" biofilms most similar to *Geobacter psychrophilus* differed by one base at position 118 (Thymine for "River" and Adenine for "Church Bay"), suggesting that both biofilms may have been dominated by different isolates of *Geobacter psychrophilus* (initially present in the inoculum). This could explain the different voltammogram shapes of the two biofilms (Figure 2a,b). The biofilm enrichment with psychrophilic microorganisms might be a consequence of the inoculum and/or the enrichment temperature. *G. psychrophilus* was previously shown to dominate anodic communities in MFCs operating at about 20°C (Liu et al., 2012; Lu et al., 2011), while other species such as *G. sulfurreducens* may predominate at temperature higher than 30°C (Miceli et al., 2012; Yoho et al., 2014), that would not allow a psychrophilic organism to thrive. The selective temperature is therefore likely to affect the composition of the anodic communities.

The "Crater Rim" biofilm was also dominated by *Geobacter* sp. (10 clones out of 17). Among the *Geobacter* species identified, 6 were similar at 97% to *Geobacter psychrophilus*. The second group was similar at 99% to *Desulfovibrio intestinalis* (3 clones out of 17). Clones most closely related to *Proteiniphilum* sp., *Pseudomonas* sp., *Clostridium* sp. and *Alcaligenes* sp. were each represented by a single DNA sequence (Figure 3). Amongst the species present (differing from *Geobacter* sp.), one or more may be responsible for the redox active species detected by cyclic voltammetry in Figure 2c.

It is interesting that three very different environments from separate geographical localities: a wet saline environment by an estuary; a wet freshwater environment by a lowland river and dry soil on a hillside, provided exactly the same dominant species after MFC selection. It suggests that *Geobacter psychrophilus* is widespread in soils, even those that do not provide a continuously anaerobic environment, and is better at growing in MFCs in these conditions than other bacteria present. Therefore, *G. psychrophilus* is "everywhere", as predicted by Baas-Becking (1934), but the environment does select where different strains will thrive. Even though the soil samples were collected in very different overall environments, each sample likely contained many micro-environments, many of which were identical. Consequently, they may be inhabited by the same bacteria. These results differed from Miceli et al. (2012) who found that bacteria related to the genus *Geobacter* dominated only two of the seven biofilms selected from diverse inocula and producing a high current on cylindrical graphite rods poised at -0.30 V vs Ag/AgCl. The voltammograms of the seven biofilms all showed sigmoidal waves, suggesting that direct electron-transfer mechanisms were involved. None of their seven biofilms showed mediated electron transfer as observed

for “Crater Rim” (Miceli et al., 2012), maybe because none were selected from an aerobic dry soil. The unique *Geobacter*-dominated community of “Crater Rim” biofilm suggests that the potential capacity for anode respiration is widespread even in environments that we might expect would be frequently exposed to oxygen.

3.2 Effect of electrode surface modifications on the selection of *Geobacter*-dominated biofilms

Two MFCs were set-up per treatment using a three-electrode configuration with the modified working electrode (-COO^- or -SO_2NH_2) poised at -0.36 V against the Ag/AgCl reference electrode. The same inoculum, water-saturated soil, was used for all the MFCs and they produced a similar amount of current over the course of the selection phase. The biofilms growing on the SO_2NH_2 electrode had the fastest start-up time with a positive current after 2.8 ± 1.1 days, compared to 6.3 ± 1.3 days for the COO^- biofilms. Although the SO_2NH_2 modification led to the fastest start-up time, it had the lowest maximum power output, while the biofilm attached to the COO^- electrode delivered the highest power density (Figure 4). The MFCs with a modified electrode had a maximum power density higher than the one with an unmodified electrode.

Modifying an electrode with SO_2NH_2 groups is likely to have a beneficial effect on the initial adhesion of bacteria to the electrode as the exoelectrogenic bacteria colonized the SO_2NH_2 electrode faster than the COO^- electrode according to their start-up times. The sulfonamides are known to have antibacterial properties when free in solution (Florestano & Bahler, 1952). Their activity depends on their lipophilicity, which determines their ability to get inside the bacterial cell, and their ionization at physiological pH. Once inside the cell, they act as competitive inhibitors of

dihydropteroate synthase (DHPS), an enzyme present exclusively in bacterial cells, resulting in the depletion of folic acid stores leading to failure of purine and thymine nucleotides biosynthesis and eventually inhibiting DNA synthesis (Valderas et al., 2008). In this case, the sulfonamides groups did not have an antibacterial activity as they were grafted on the electrode surface. However, their lipophilicity may have accelerated the attachment of bacterial cells to the electrode by penetrating the bilayer structures of phospholipids of the outer-membrane. This would have resulted in the more rapid development of the anode-respiring biofilm and so the production of electricity, explaining the faster start-up time. However, the biofilms growing on SO_2NH_2 did not perform as well as the COO^- biofilms as shown by the lower power density (Figure 4).

It was anticipated that the carboxylates groups would repulse negatively charged bacteria due to their negative charge at pH 7 and so slow down the start-up time or even the current production (Picot et al., 2011). The COO^- MFCs had a start-up time twice as long as the SO_2NH_2 MFCs, but it showed the highest power density and the highest catalytic current (Figure 5), suggesting that the electron transfer from the biofilm to the electrode was very efficient. These results are at variance with those of Picot et al. (2011) who showed that a negatively charged electrode surface led to a drop of power densities compared to an unmodified electrode. However, bacteria poorly colonized their electrode modified with benzylcarboxylate groups. Picot et al. (2011) explained their results by an electrostatic repulsion between the electrode surface and negative charge of bacteria including *Geobacter* sp. Unlike Picot et al. (2011), Kuzume et al. (2013) found that carboxyl groups interact with the outermost cytochromes of *Geobacter sulfurreducens*, facilitating the heterogeneous electron transfer at the

microorganism/electrode interface. In this system, we noted that the COO^- fuel cell had the lowest internal resistance with 422 Ω against 463 Ω for the unmodified electrode and 495 Ω for the SO_2NH_2 MFC. This may be explained in part by the affinity of carboxylate groups with the outer-membrane cytochromes facilitating the electron transfer from the biofilm to the electrode.

The turnover voltammograms performed after 29 days of selection showed a sigmoid catalytic wave for the two modified electrodes. This wave is characteristic of acetate oxidation via the catalytic action of the biofilm grown on the electrode surface (Figure 5). The COO^- anode had a significantly lower midpoint potential of acetate oxidation at -0.406 ± 0 V against -0.394 ± 0.001 V vs Ag/AgCl for the SO_2NH_2 electrodes. This is consistent with a relatively faster electron transfer at the carboxylate modified electrode.

ARISA was performed at the end of the experiment, after 29 days of incubation, to check if the different electrode surface modifications selected for different bacterial communities. The two biofilms were dominated by a small number of taxa, as shown by the small number of peaks on their ARISA electropherograms (Figure S1a,b). They both had similar dominant bacterial communities with the same four dominant peaks present in each of their ARISA profiles at 619 bp, 633 bp, 679 bp and 703 bp. Those peaks were previously observed in the ARISA profiles of *Geobacter*-dominated biofilms selected at -0.36 V vs Ag/AgCl (Commault et al., 2013). In spite of being selected on different electrode surfaces, the biofilms had similar dominant communities. The analysis of the ARISA profile data using the Bray Curtis similarity matrix revealed that while no large difference amongst the dominant taxa was observed in the two types of biofilms, there were nevertheless consistent differences in community structure

(Figure S1c). The Bray Curtis similarity matrix takes more base pairs into account than can be visually detected with an electropherogram, and showed that the biofilms from the same electrode modification were more similar to each other than to biofilms from the other modification. The results from the ARISA profiles and the similarity matrix suggested that there were small but consistent differences in the dominant communities of the COO^- and SO_2NH_2 biofilms.

All 16S rRNA gene clones (13/13) of the biofilms selected on the SO_2NH_2 modified electrodes were identified as being similar at 96% to *Geobacter psychrophilus*. As the COO^- biofilms were dominated by bacterial OTU with the same ITS length as SO_2NH_2 biofilms (Figure S1a), we assumed that COO^- biofilms were dominated by *Geobacter psychrophilus* too.

We anticipated that changing the electrode surface properties could select for different bacterial communities. For instance, a negatively charged electrode surface at pH 7 (e.g., -COO^- modification) should electrostatically repulse bacteria and may result in different bacterial communities than neutral electrode surface modification (e.g., -SO_2NH_2 modification). The electrode modifications did not influence the composition of the most dominant species in the selected biofilms, as their ARISA profiles were similar. However, consistent subtle changes in community composition were detected by Bray Curtis similarity analysis of ARISA profile data. These results are different from the results of Picot et al. (2011), who found using fluorescence *in situ* hybridization (FISH, probe Geo1A), that biofilms selected from domestic wastewater on anode modified with benzylcarboxylate groups (functional group -COO^-) had less bacterial cells than biofilms selected on positively charged surfaces (modified with aryl diazonium salts or 4-benzyl triphenylphosphonium diazonium), and only few of them

belonged to the *Geobacter* subgroup (Picot et al. 2011). However, Guo et al. (2013) showed that differences in biofilm communities were attributed to differences in hydrophilicity or hydrophobicity of electrode surfaces more than to differences in the charge of the electrode modification groups. They showed that glassy carbon surfaces modified with $-CH_3$, -OH, $-SO_3^-$, or $-N^+(CH_3)_3$ functional groups by electrochemical reduction of aryl diazonium salts all led to the selection of biofilms dominated by *Geobacter* sp., with the positively charged and hydrophilic surfaces being more selective to electro-active microbes. Their FISH results showed that at the electrode interface, the relative *Geobacter* abundance on the hydrophobic surface ($-CH_3$) was only about half of that of the biofilms on hydrophilic surfaces ($-N^+(CH_3)_3$, -OH, and $-SO_3^-$). Hence, Guo et al. (2013) hypothesized that the surface hydrophobicity affects the initial attachment of *Geobacter* sp., and so the subsequent biofilm development. The two functional groups tested in the present study probably increased the hydrophilicity of the graphite electrode surface. The increase of hydrophilicity compared to the unmodified electrode, would have favoured the attachment of *Geobacter* sp.

4. Conclusion

The different inocula and electrode surface modifications tested all selected for *Geobacter*-dominated biofilms. However, there were major differences in the biofilm communities selected from the different inocula and small but consistent differences in the dominant communities of the COO^- and SO_2NH_2 biofilms. The two factors also affected the electrochemical properties of the biofilms.

Because of the dominance of *Geobacter* sp. in electroactive biofilms, the use of different inocula or anode surface modifications is unlikely to lead to the discovery of

new anode-respiring bacteria, but it could shed light on new metabolic pathways for higher current production in MFC.

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Figure Captions

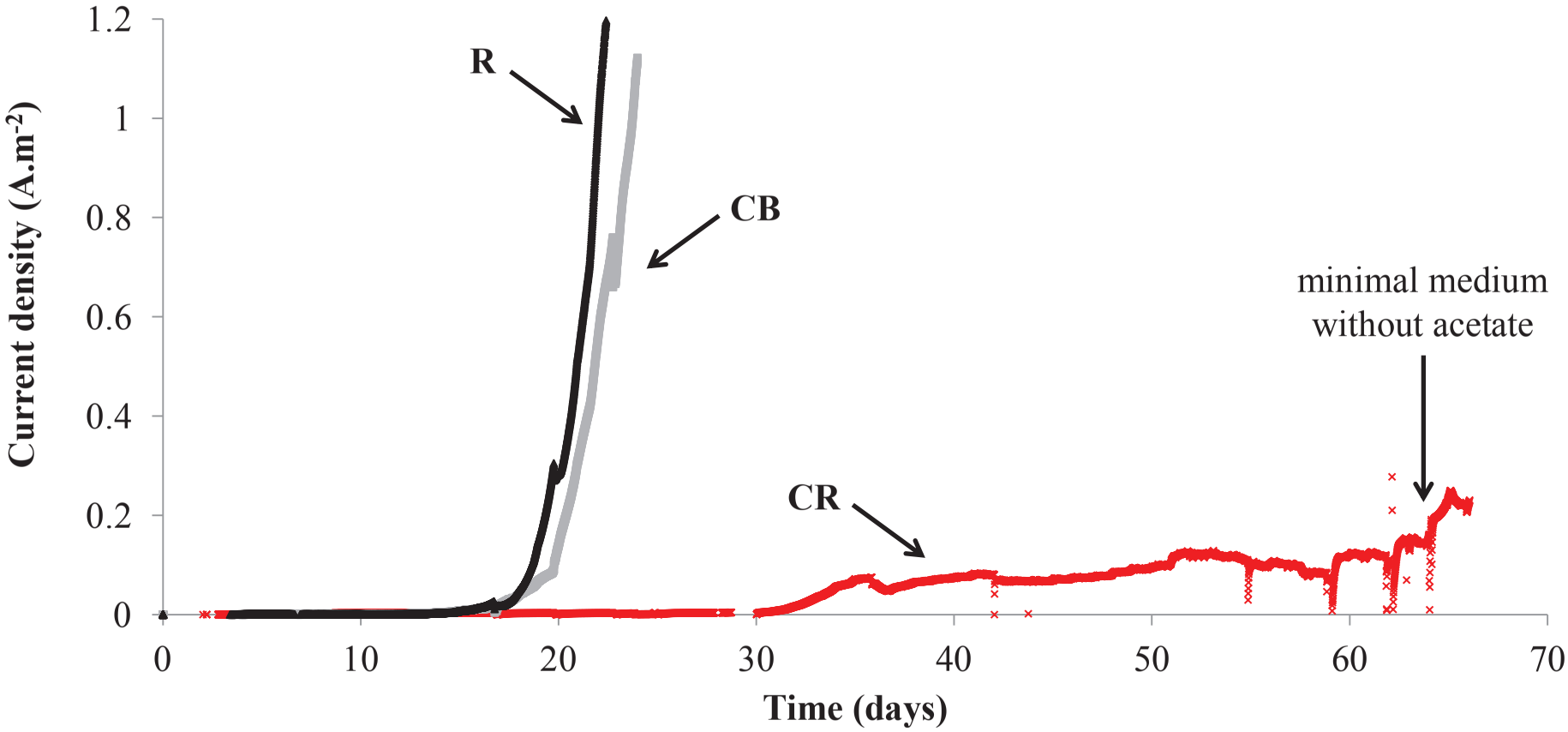
Figure 1. Current generated by the MFCs with different inocula over time. Dry soil from "Crater Rim" (CR, red cross), saline soil from "Church Bay" (CB, grey square) and wet soil from "Halswell River" (R, black triangles).

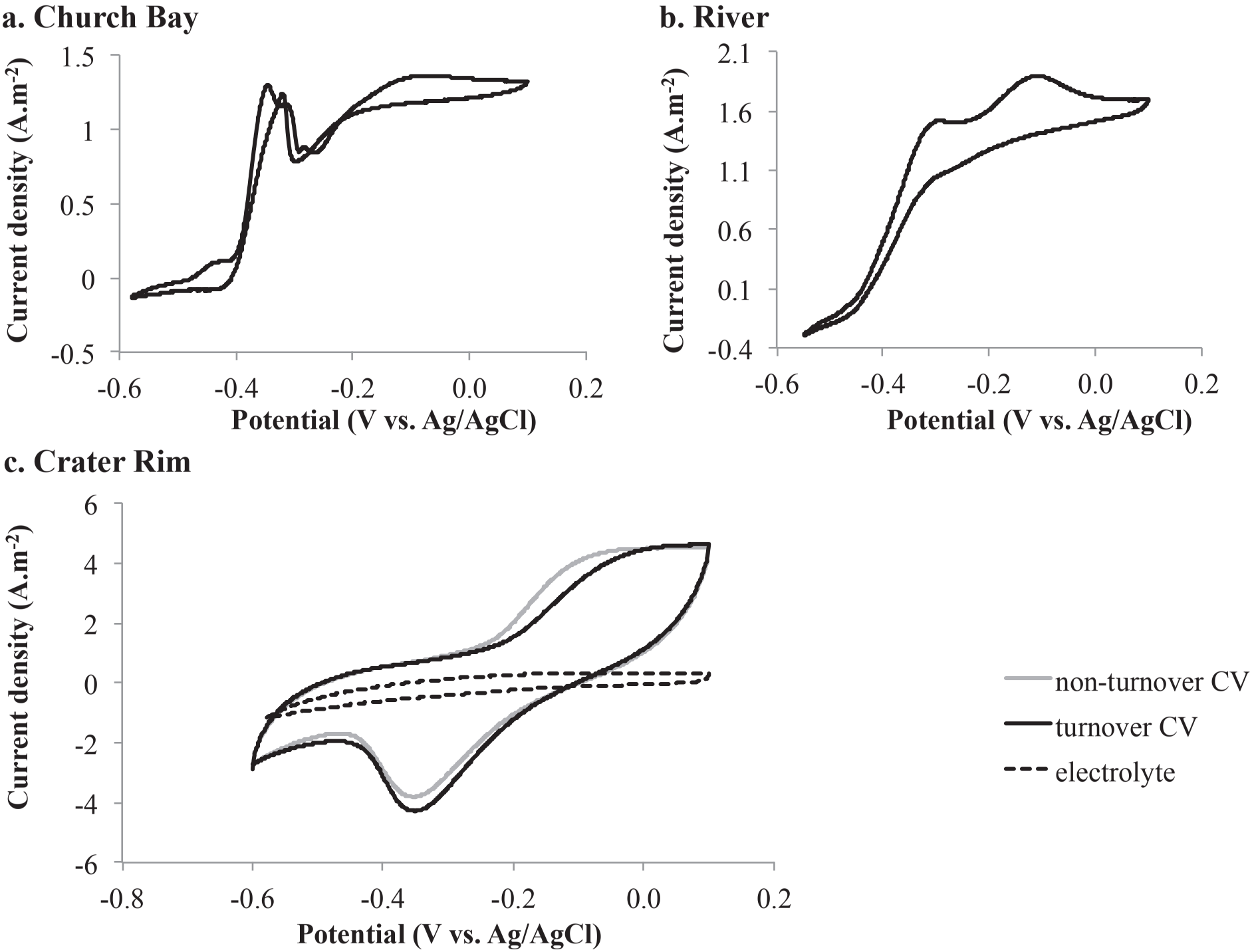
Figure 2. Cyclic voltammograms of the biofilms selected from the three different inocula after 29 days of enrichment (66 days for "Crater Rim"). **a.** and **b.** Turnover voltammograms of "Church Bay" and "River biofilms". **c.** Voltammograms of "Crater Rim" biofilm in turnover conditions (black lines), non-turnover conditions (grey line) and the electrolyte of "Crater Rim" MFC (black dashed line). The 3rd and 4th segments are shown on the graph.

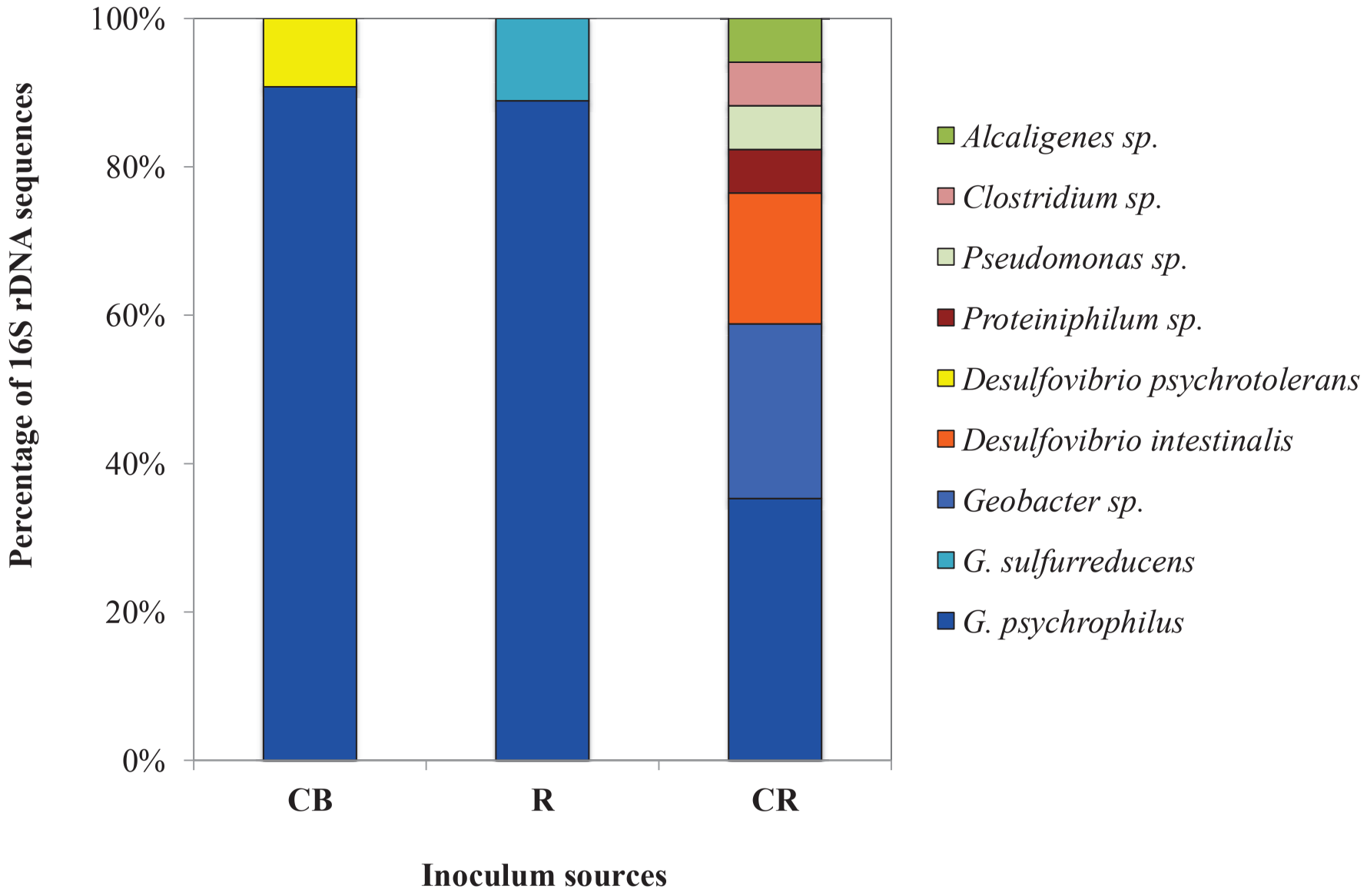
Figure 3. Microbial community distribution for anodic communities enriched from three different inocula: Church Bay (CB), River (R) and Crater Rim (CR).

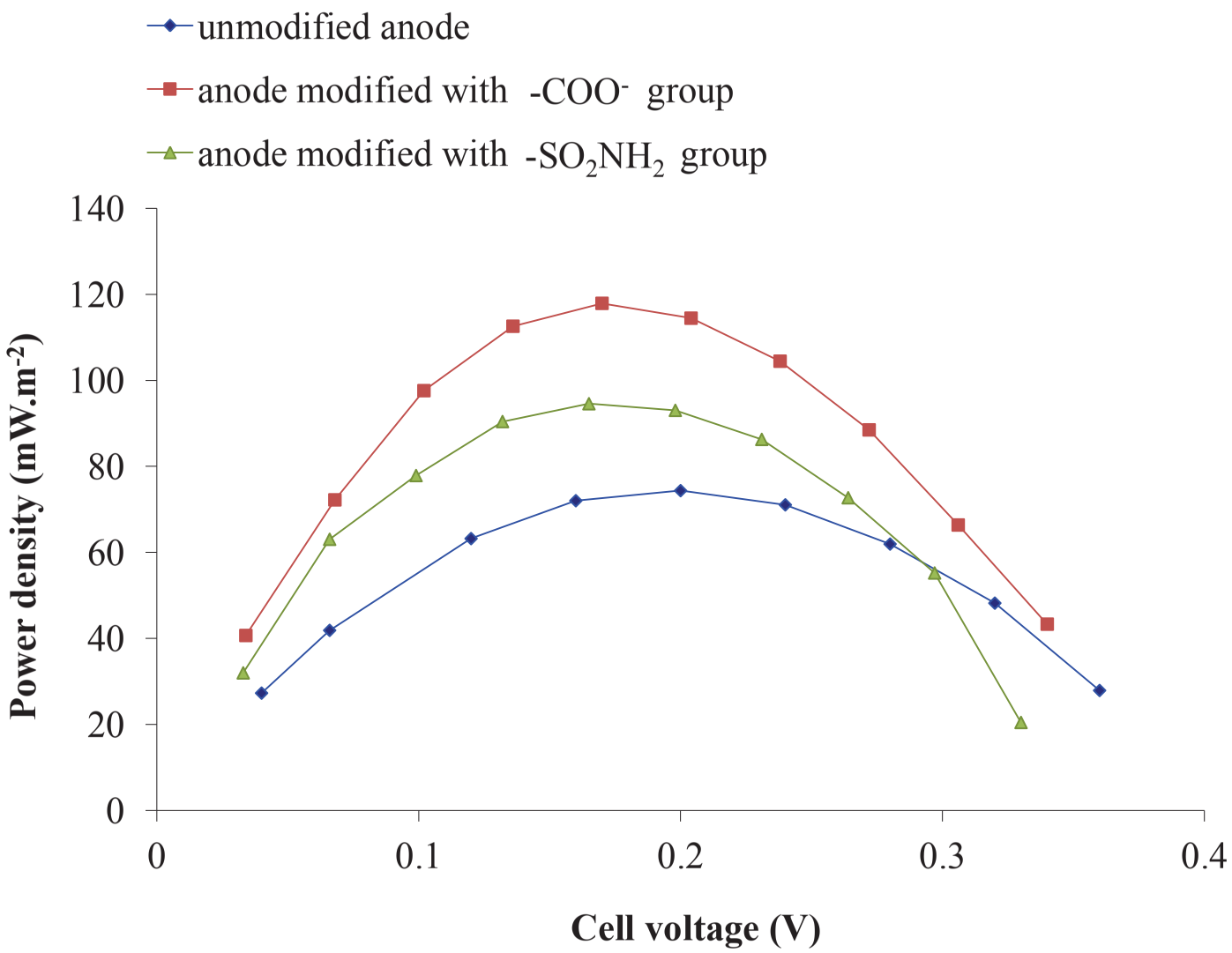
Figure 4. Power density curves for modified and unmodified anodes performed 29 days after inoculation. The power density curve of a 29 day-old "unmodified" anode grown in the same MFC configuration, at the same potential with the same inoculum is given for comparison (data from Commault et al. (2015)). This graph shows the data of one representative sample per treatment.

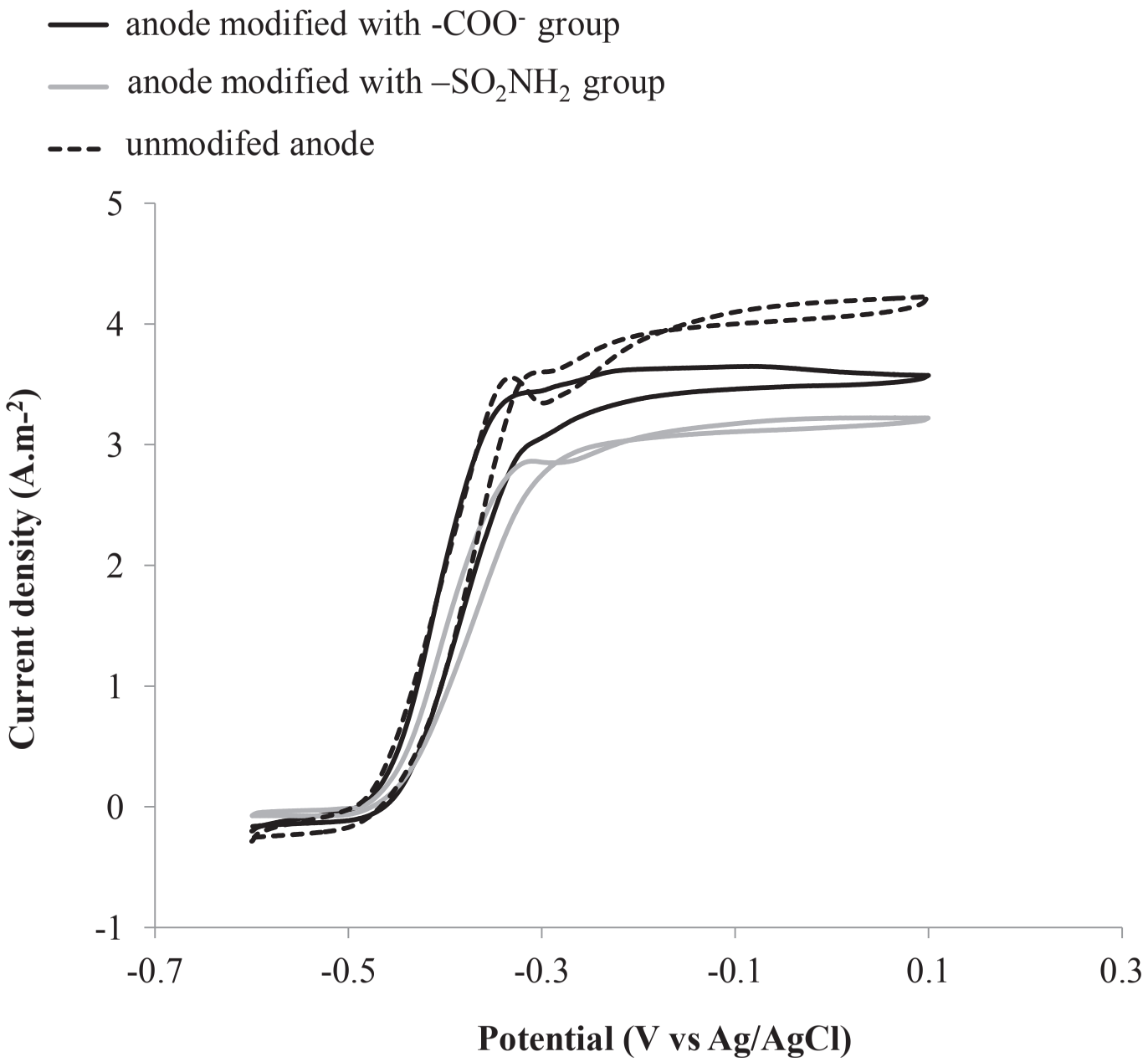
Figure 5. Turnover voltammograms of an unmodified anode and the modified electrodes after 29 days of selection in MFCs. Data are an average of the 3rd and 4th segments of two replicates per treatment. The voltammogram of an "unmodified" anode grown in the same MFC configuration, at the same potential with the same inoculum is given for comparison (data from Commault et al. (2015)).


Current density (A.m-2)
1.2
1
0.8
0.6
0.4
0.2
0
R
CB
CR
minimal medium
without acetate
0
10
20
30
40
50
60
70
Time (days)


a. Church Bay
Current density (A.m-2)
1.5
1
0.5
0
-0.5
-0.6
-0.4
-0.2
0.0
0.2
Potential (V vs. Ag/AgCl)
b. River
Current density (A.m-2)
2.1
1.6
1.1
0.6
0.1
-0.4
-0.6
-0.4
-0.2
0.0
0.2
Potential (V vs. Ag/AgCl)
c. Crater Rim
Current density (A.m-2)
6
4
2
0
-2
-4
-6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
Potential (V vs. Ag/AgCl)
non-turnover CV
turnover CV
electrolyte


Percentage of 16S rDNA sequences
100%
80%
60%
40%
20%
0%
CB
R
CR
Inoculum sources
Alcaligenes sp.
Clostridium sp.
Pseudomonas sp.
Proteiniphilum sp.
Desulfovibrio psychrotolerans
Desulfovibrio intestinalis
Geobacter sp.
G. sulfurreducens
G. psychrophilus


unmodified anode
anode modified with -COO- group
anode modified with -SO2NH2 group
Power density (mW.m-2)
140
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
Cell voltage (V)


anode modified with -COO- group
anode modified with –SO2NH2 group
unmodifed anode
Current density (A.m-2)
5
4
3
2
1
0
-1
-0.7
-0.5
-0.3
-0.1
0.1
0.3
Potential (V vs Ag/AgCl)

Highlights

- Dry soil MFC had longest start-up time (28 d) and poor current output
- COO^- anodes had longest start-up times (6.3 d) but highest power output (118 mW/m^2)
- All biofilms selected were dominated by *Geobacter* sp.
- *Geobacter* sp. is widespread in soils, even those frequently exposed to oxygen
- *Geobacter* is very much better at growing in MFC conditions than any other bacteria