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Influence of activated carbon on performance and microbial communities in the treatment of solvent pollutant mixtures in a continuous stirred tank reactor

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Abstract

The influence of the addition of granular activated carbon (GAC) to the anaerobic treatment of wastewater with a mixture of ethanol and 1-ethoxy-2-propanol (E2P), which are typical pollutants from flexographic/printing industry, was evaluated. For this purpose, reactor performance and microbial community were assessed in two continuous stirred tank reactors (CSTRs), i.e. control and GAC supplemented. Both reactors, seeded with anaerobic granular biomass, showed similar global performance (RE>93%), except after organic load shocks and after E2P addition. GAC supplementation substantially enhanced the response of the biological system to organic load shocks and reduced the acclimation period for E2P degradation. This improvement was found to be related to the effect of GAC on the microbial community which, in turn, is related to an increase in the degradation rate of intermediates (acetone and isopropanol). The microbial community was analysed through denaturing gradient gel electrophoresis and high-throughput sequencing. Both methods showed an evolution from the inoculum to the final time point and considerable enrichment in microorganisms involved in direct interspecies electron transfer, such as *Geobacter* species in the final biomass samples, especially in the biomass attached to GAC.

1. Introduction

Volatile organic compounds (VOCs) emissions must be controlled according to the environmental regulations of many countries, including those of the European Union.[1] The flexographic industries are one of the major contributors to these emissions, which are characterised by a high air flow and a low pollutant concentration of biodegradable solvents (mainly ethanol) and to a lesser extent retarders such as glycol ethers (i. e. 1-ethoxy-2-propanol).[2] Recently, a new technology based on an anaerobic bioscrubber was described as a high-efficiency technique to treat these emissions and convert these hydrophilic pollutants into bioenergy,[3] in which VOCs are transferred from the air to the water in a scrubber, and then into an anaerobic reactor where they are transformed into biomethane.[4]

Some authors have investigated the anaerobic treatment of solvents from the flexographic sector. Ferrero *et al.*[5] showed that an expanded granular sludge bed (EGSB) reactor and an anaerobic hybrid reactor (AHR) showed a similar performance (RE ≈ 95%) when treating a mixture of ethanol, 1-ethoxy-2-propanol (E2P) and/or 1-methoxy-2-propanol (M2P). Torres *et al.*[6] successfully achieved granulation in Upflow Anaerobic Sludge Blanket (UASB) reactors by treating a mixture of ethanol, ethyl acetate and E2P with RE higher than 92%. Regarding the associated microbial communities, these authors found using different molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) and high-throughput sequencing, that the genus of *Geobacter* was predominant in the bacterial population.[7] This was also observed by Bravo *et al.*,[3] who used DGGE to analyse the microbial evolution of an EGSB reactor from an on-site pilot scale plant treating real emissions from the flexographic industry. These findings indicate that these species could play an important role in the anaerobic degradation of solvents from flexographic facilities, so enrichment with these kinds of microorganisms could improve the performance of anaerobic reactors treating solvents from the printing industry. In this context, it has to be highlighted that the *Geobacter* electroactive bacteria are usually involved

in direct interspecies electron transfer (DIET) in methanogenesis.[8] This interaction can be carried out in a number of ways, i.e. via direct contact between electron acceptor and the redox-active proteins on the outer–membrane surface (through biological components like c-type cytochromes) and/or via electrically conductive filaments, so-called nanowires or e-pili.[9-12] Some authors have also shown that DIET can be facilitated by supplementing with abiotic conductive materials such as iron-oxides[13] or granular activated carbon (GAC).[14] Some additional advantages have been reported for reactors supplemented with conductive materials. Lag times to produce methane were reduced by up to 75% in reactors supplemented with conductive materials.[15] Lee *et al*.[16] showed in a CSTR reactor supplemented with GAC and fed with acetate a higher percentage of acetate conversion into methane than in a non-supplemented reactor (97% vs. 66%). Additionally, these authors reported that the biomass attached to the GAC had a specific methane production 3.7 times higher than the biomass in the bulk solution. Lee *et al*.[16] studied the archaeal diversity and reported that the family *Methanospirillaceae* had considerably increased its relative abundance in the biomass attached to the GAC. The effect of the particle size of the conductive material was investigated by Xu *et al.*[17] using two particle sizes (10-20 mesh GAC and 80-100 mesh powdered activated carbon, PAC). This work showed very similar results for the GAC and PAC reactors. Supplementation with GAC and PAC lead to enhanced biogas production, shorter start-up periods and greater stability against organic loading shocks. Zhao *et al.*[18] showed that a reactor supplemented with carbon cloth treating butanol resisted low pH (6), and recovery was faster when the pH was reverted back to neutrality. Furthermore, Zhao *et al.*[19] reported that an UASB supplemented with conductive materials (biochar, carbon cloth and graphite) resisted high organic loading rates (OLR) (12.3 kg COD m^{-3} d^{-1}).

In practically all these studies, non-granular sludge from an anaerobic digester was used. Only one study carried out in a batch reactor reported on the use of aggregates from a methanogenic digester, obtaining a methane production rate 2.5-fold faster with GAC than in the control

87 conditions without GAC.[14] Therefore, the influence of granular biomass on DIET mechanism
88 enhancement and the benefits of adding conductive materials during the anaerobic degradation
89 of solvent pollutants is not well-established, especially in continuous reactors. Additionally, in
90 most previous studies, the substrates to be degraded were ethanol and/or short chain fatty
91 acids, but the effect on degradation of other typical printing solvent compounds, such as glycol
92 ethers, has not been reported so far.

93 As mentioned above, high throughput sequencing and DGGE have been frequently used as
94 techniques for microbial characterization in anaerobic reactors. Usually, both molecular tools
95 provide information that can coincide to some extent; nevertheless, some remarkable
96 differences should be pointed out. High throughput sequencing can obtain a complete
97 identification of the microbial communities but, unfortunately, a significant amount of
98 metagenomic reads have not yet been identified. In addition, the higher abundance of *Bacteria*
99 in comparison to *Archaea*,[7,20] can lead to mask the identification of archaeal microorganisms.
100 DGGE technique provides an immediate display of the microbial constituents but due to its
101 inherent practical limitations, which can cause a relatively low high-throughput, DGGE analysis
102 is often unsuitable for the identification and quantification of microorganisms in biodiverse
103 samples, with the resultant loss of valuable information.[21] The combined use of both techniques
104 could overcome these drawbacks.

105 In this context, the main goal of this work was to determine the effect of adding GAC on the
106 behaviour of a continuous stirred tank reactor (CSTR) in the treatment of a representative
107 mixture (ethanol:E2P) of flexographic solvents. For this purpose, the global performance,
108 methane production and microbial community structure (via DGGE and high throughput
109 sequencing) of a GAC supplemented reactor have been compared to a CSTR without GAC
110 addition under the same operation conditions.

111

2. Materials and Methods

2.1 Reactor setup

The experiment was carried out over a period of more than 3 months in two CSTRs with a total volume of 2 L and an effective volume of 1.6 L. The scheme of the reactors is shown in Figure 1. Control (R1) and GAC supplemented (R2) reactors were seeded with 16 g of volatile suspended solids (VSS) of an anaerobic granular sludge taken from a brewery wastewater treatment plant. The main physical properties of the granular inoculum[7] were: 2.22 ± 0.94 mm of average particle diameter, 8.2 ± 1.2 mg of total solids g wet w^{-1}, and 7.4 ± 0.1 mg of volatile solids g wet w^{-1}. Additionally, the GAC reactor (R2) was supplemented with 16 g of granular activated carbon (Darco® 12x20 mesh, surface area of 650 m^2 g^{-1}). Reactors were operated at 25°C by means of a thermostatic water bath (Memmert GmbH + Co. KG, Germany), simulating the operational condition of a full-scale bioscrubber.[3] Inlet and outlet flows were fixed at 0.2 L d^{-1}, obtaining a hydraulic retention time (HRT) of 8 days. Synthetic wastewater was pumped into the reactors through a multichannel peristaltic pump (Reglo ICC, Ismatec ©, Germany). Feed was buffered with 5 g L^{-1} bicarbonate, and Ca^{2+} and Mg^{2+} were introduced to the feed with a constant concentration of 40 mg L^{-1}. The rest of the macro and micronutrients were dosed in proportion according to Lafita el al. [22] and it can be found in Supplementary Material (Table S1) . Reactors were stirred discontinuously, i.e. 30 s on/30 s off (100 rpm). The stirring procedure was selected both to avoid that granular sludge (or activated carbon) flowed out the reactor and to maintain the integrity of the granules.

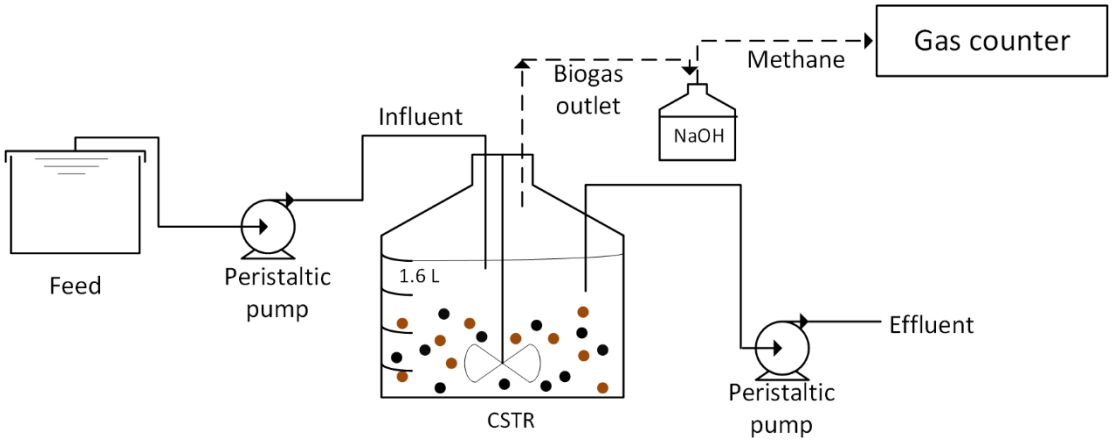


Figure 1. Scheme of the experimental setup.

Experiment was divided into seven stages (from S1 to S7), as shown in Table 1. During the first three stages (S1 to S3) only ethanol was fed as carbon source and the OLR was progressively augmented from 1.25 to 6.25 g COD L^{-1} d^{-1}, by increasing the influent concentration of COD. Then, from S4 to S7, glycol ether (E2P) was added to the feed in an ethanol:E2P mass ratio of 8:2. The organic loading rate (OLR) during these stages was progressively increased from 4.38 g COD L^{-1} d^{-1} (S4) to 9.38 g COD L^{-1} d^{-1} (S7), as can be found in Table 1.

Table 1. Organic loading rate (OLR) and feeding composition in each stage.

Stage	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
Time (d)	0-13	14-34	35-41	42-49	50-84	85-96	97-105
OLR (kg COD m^{-3} d^{-1})	1.25	3.75	6.25	4.38	6.25	7.50	9.38
Ethanol:E2P*	10:0	10:0	10:0	8:2	8:2	8:2	8:2

*Mass ratio

2.2 Analytical methods

The COD concentration was analysed according to standard methods.[23] Conductivity and pH were measured using a multimeter (Multi 340i, WTW, Germany). Total volatile fatty acids (VFA), expressed as concentration of acetic acid equivalent, and alkalinity concentration in the effluent of the reactors were determined by the five-point acid-base titration method[24] using a titrator

147 (848 Titrino Plus, Metrohm, Switzerland). The effluent's by-products and solvents were
148 identified by gas chromatography-mass spectrometry (5973 N MS/GC Agilent Technologies,
149 Spain). The concentration of solvents and by-products were determined through gas
150 chromatography (Agilent GC 7890A, Spain) equipped with a flame ionisation detector (FID),
151 where a Restek Rtx-VMS column (30 m x 0.25 mm x 1.4 µm) was installed to separate the organic
152 compounds and helium was used as the carrier gas at a flow rate of 1.3 mL min^{-1}. The methane
153 flow rate was continuously monitored using an Automatic Methane Potential Test System
154 (AMPTS II, BioProcess Control, Sweden) after removing the H_2S and CO_2 contained in the biogas
155 by passing the biogas through an NaOH solution.

156 **2.3 Microbial community analysis**

157 Samples of biomass were collected at beginning and at the end of the experiment. Biomass
158 samples from R2 were differentiated into granules (G-R2) and the biomass attached to the
159 activated carbon (GAC-R2). DNA was extracted using the PowerSoil DNA isolation kit (MoBio
160 Laboratories, USA) according to the supplier's protocol. DNA extraction was checked by
161 measuring the DNA concentration using a Nanodrop® (Thermo Scientific, USA). The extracted
162 DNA was stored at -20°C until further analysis.

163 **2.4 DGGE protocol**

164 The V3 region of the 16S rDNA gene of *Bacteria* was amplified through the primer pair F357-GC
165 and R518,[25] while the primer set F787-GC and R1059[26] was used to amplify the 16S rDNA of
166 *Archaea*. Conditions for PCR were: 20 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 0.5 min,
167 then 10 cycles in case of *Bacteria* amplification or 15 cycles for *Archaea* amplification of 94°C for
168 1 min, 55°C for 1 min, 72°C for 0.5 min, and a final extension of 72°C for 10 min. The PCR product
169 was electrophoresed on a 2% (w/v) agarose gel to check for proper amplification. DGGE was
170 conducted using the KuroGel Verti 2020 DGGE system (VWR International Eurolab, Spain),
171 where 20 µL of the PCR product was separated on an 8% acrylamide gel with a linear denaturing

gradient gel from 35% to 55% at 60°C and 100 V for 840 min. DGGE gels were visualised using the MiniBIS Pro System (DNR Bio-Imaging System Ltd., Spain). Predominant bands were excised and reamplified using the same PCR conditions previously reported and the PCR products were purified with an mi-PCR purification kit (Metabion, Germany) and sequenced with an automated DNA analyser (3730 KL DNA Analyser, Applied Biosystem, Spain). Finally, the obtained sequences were compared with those available from the NCBI GenBank using BLAST software.

In order to facilitate the analysis of the DGGE gel, a marker with DNA from different strains was constructed for *Bacteria* and *Archaea*. To do this, the V3 region of the 16S rRNA gene of different strains was also amplified using the conditions mentioned previously. For the Bacteria marker, the following strains were used: *Pseudomonas aeruginosa*, *Escherichia coli*, *Thiobacillus thiooxydans* and *Xantobacter autotrophicus*. For the *Archaea* marker, the following strains were used: *Methanocorpusculum bavaricum*, *Methanosarcina mazei* and *Methanobrevibacter smithii*. After amplification, DNA was purified and quantified and then mixed to a concentration of 5 ng μL^{-1}.

2.5 High-throughput sequencing analysis

The V4 hypervariable region of the 16S rRNA gene was amplified using the primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3'). After that, the amplicons were sequenced with the MiSeq system (Illumina, San Diego, USA). Finally, the sequences were analysed with bioinformatics tools and screened and trimmed by using Quantitative Insights Into Microbial Ecology (QIIME) software, with a sequence length of 200 and a mean quality score cut-off of 25 nt.

3. Results

3.1 Comparison of reactor performance

The global removal efficiency and the evolution of the VFA concentration in the effluent of both reactors are shown in Figure 2a. As can be observed, the pollutant removal was almost complete during the first two stages (S1 and S2), when only ethanol was used as the substrate.

The influence on the pollutant removal of the solvent adsorption on GAC (R2) can be considered negligible after the first 8 hours of operation, taking into account both the pollutant loading rate and the adsorption capacity of ethanol aqueous mixtures on GAC at similar operational conditions, with reported values ranging from 0.4 to 0.1 g of total (reversibly and irreversibly) adsorbed pollutant per g of GAC. [27,28].

In stage S3, when the OLR was increased to 6.25 g L^{-1} d^{-1}, the global RE slightly decreased to 96% in both reactors, leading to a transitory acid accumulation (up to 1802 and 1296 mg acetic acid equivalent L^{-1} in the control and GAC reactors, respectively) related to a kinetic limitation in the methanogenic step. Global RE increased after just 6 days in the GAC reactor (R2) to practically 100%, while in control reactor (R1) remained at 97% (915 mg acetic acid equivalent L^{-1}). This behaviour of the GAC supplemented reactor may be related to the effect against shock loads of GAC, as indicated Xu et al.,[17] recovering its performance faster than the control reactor. Then, E2P was introduced (S4), but the OLR was decreased to 4.38 g COD L^{-1} d^{-1} in both reactors in order to avoid an acid crash in the control reactor. In this stage, the global RE in the control reactor was recovered to nearly complete removal. In the following stage (S5), when the OLR was increased again to 6.25, the global RE gradually decreased until day 64 down to 94% in both systems. Then (day 65), the global RE recovered in the GAC reactor to 97% and remained around these values, while in control reactor, the RE ranged between 93% and 94%. When the OLR was increased to 7.25 g COD L^{-1} d^{-1} (S6), no noticeable change was observed in the global RE for the GAC reactor whereas it increased to 96% in the control reactor. This decrease (50-65 days) and subsequent recovery (78-89 days) in the global RE could be linked to biomass adaptation to E2P. Ferrero *et al.*[5] showed that an adaptation period of around 30 days is needed to achieve the

maximum RE of this compound in an EGSB reactor. Thus, it seems that GAC addition would shorten to some extent this adaptation period to degrade E2P. In the final stage (S7), the OLR was increased to 9.38 g COD L^{-1} d^{-1}, resulting in a decrease in the global RE and acid accumulation in both reactors, which resulted in acidification of the reactors.

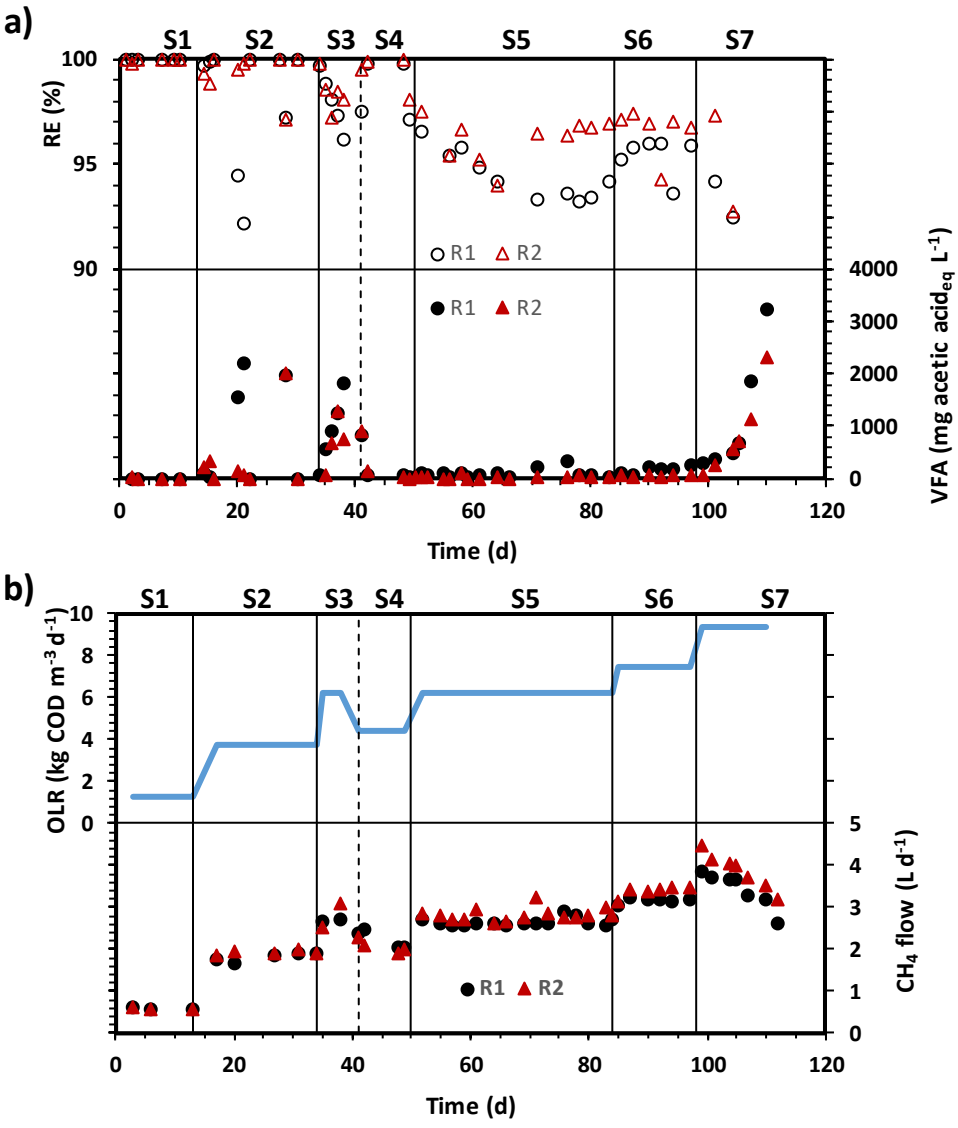


Figure 2 a) Evolution of RE in both reactors and the VFA concentration in the effluent of both reactors. b) Evolution of OLR and the methane flow in each reactor during the experimental period. Circles refer to the control reactor (R1) while triangles refer to the GAC-supplemented reactor (R2).

Methane production was very similar for both reactors before the introduction of E2P in stage S4 (Figure 2b), and methane flow increased proportionally to the OLR increase. Only on sporadic days was methane production slightly decreased in the control reactor, usually associated with a small increase in the VFA concentration. After E2P introduction, the GAC reactor produced slightly more methane than the control reactor, which could indicate higher degradation of this compound. Finally, in the last stage (S7), methane production increased up to 3.9 and 4.5 L d^{-1} in R1 and R2, respectively, but decreased over time due to the increasing VFA accumulation, which caused a progressive inhibition of methanogenesis.

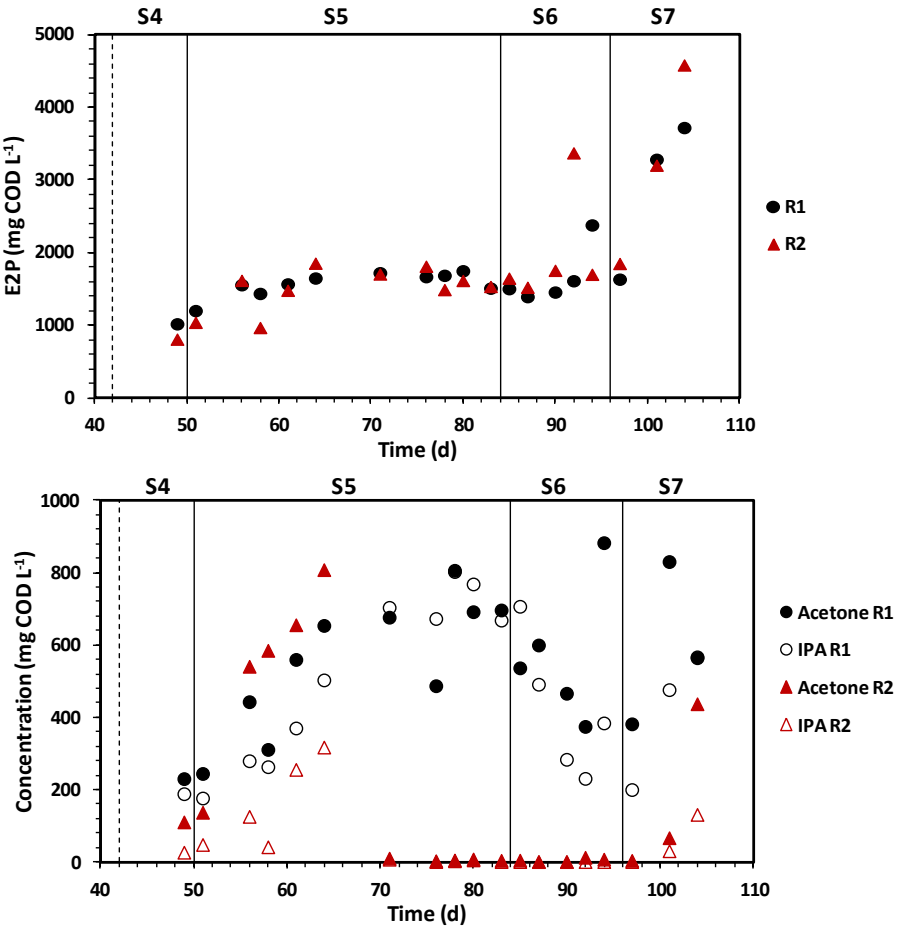


Figure 3. a) Evolution of the E2P concentration in the effluent of R1 and R2. b) Evolution of intermediate compounds in the effluent of both reactors.

241 The evolution of the E2P effluent concentration (Figure 3a) was similar in both reactors,
242 stabilising around to 1600 mg COD L^{-1} during S5 and remaining quite stable until the end of S6,
243 leading to an E2P RE of around 87%. Finally, in the last stage (S7), the E2P effluent concentration
244 gradually increased, reflecting that the steady state was not achieved. The high VFA
245 accumulation was concomitant with the decrease in E2P cleavage, leading to similar E2P effluent
246 concentrations for both reactors. Therefore, it seems that GAC addition did not improve the first
247 step (the ether cleavage) of the degradation of E2P. This could be associated with the
248 microorganisms involved in DIET, because these microorganisms prefer to use fatty acids or low
249 molecular weight alcohols rather than glycol ethers. E2P is degraded to ethanol and acetone,
250 which can be transformed into isopropanol in an anaerobic reactor, as previous studies have
251 shown.[5] In this regard, these intermediate products (acetone and isopropanol) of E2P
252 degradation were detected and quantified in this experimental study (Figure 3b). The acetone
253 and isopropanol concentrations in the effluent of the control and GAC supplemented reactors
254 gradually increased until day 68, from which point neither isopropanol nor acetone were
255 detected in the GAC reactor effluent. Nevertheless, in the control reactor, the concentration of
256 intermediate compounds in the effluent remained constant between 700 and 800 mg COD L^{-1}
257 until day 85. During stage S5, the acetone and isopropanol concentrations progressively
258 decreased to 381 and 199 mg COD L^{-1}, respectively. This difference in the concentrations of the
259 intermediate compounds could explain why, although E2P removal was almost identical in both
260 reactors, global RE was higher in the GAC reactor than in the control reactor.

261 Since no intermediate compounds (acetone and isopropanol) were detected from day 70 in the
262 GAC reactor, in contrast to the control reactor, this seems to indicate that GAC supplementation
263 led to a reduction in the adaptation time needed to degrade the intermediate compounds. This
264 is in accordance with previous studies reporting a reduced lag time in reactors where conductive
265 materials were added.[15] Finally, in the last stage (S7), the concentration of the intermediate
266 compounds increased again due to organic load shock, but this increase was more pronounced

267 in the control reactor. The similar behaviour regarding E2P hydrolysis (ether cleavage) and the
268 evolution of different intermediate compounds indicate that E2P cleavage was carried out by
269 microorganisms that are not involved in DIET. In contrast, the degradation of the intermediate
270 compounds was likely facilitated by electro-active microorganism growth on the GAC surface.
271 Considering that ethanol is one of the intermediate compounds of E2P degradation and acetone
272 is converted into acetate, it is possible that the complete absence of both compounds in the
273 effluent of the GAC reactor was due to the growth of these microorganisms on the GAC surface,
274 which can only use certain substrates, such as fatty acids and/or alcohols. Furthermore, when
275 the intermediate compounds accumulated in both reactors, the isopropanol concentration was
276 lower in the effluent of the GAC supplemented reactor (R2). This can be explained because DIET
277 is not mediated by other electron carriers such as hydrogen or formate, and acetone in presence
278 of hydrogen can be reduced to isopropanol.

279 **3.2 Microbial community analysis**

280 **3.2.1. Denaturing gradient gel electrophoresis analysis**

281 The archaeal and bacterial DGGE results can be found in Figure 4a and Figure 4b, respectively.
282 In both figures, an evolution from the inoculum to the final samples, both granular (G-R1 and G-
283 R2) and attached (GAC-R2), can be observed, where some of the predominant bands in the
284 inoculum decreased their intensity, or completely disappeared, and others became
285 predominant. Furthermore, it is remarkable that the granular samples of both reactors (G-R1
286 and G-R2) evolved in an almost identical way in the archaeal and bacterial DGGE, appearing
287 practically the same and even with similar band intensity. However, the biomass attached to the
288 GAC (GAC-R2) showed a different pattern. In the archaeal DGGE, the band pattern was similar
289 between G-R1 and G-R2, although the intensity of some bands was slightly different (band BA1,
290 BA2 and BA6). In contrast, the bacterial DGGE pattern was substantially different in case of the
291 GAC-R2 and only a few bands with a relativity high intensity were detected, which reveals that

a very specialised microbial community had developed on the GAC surface. In samples GR1 and GR2, many bands of relatively low intensity were detected, suggesting higher microbial diversity than on the surface of the GAC (GAC-R2).

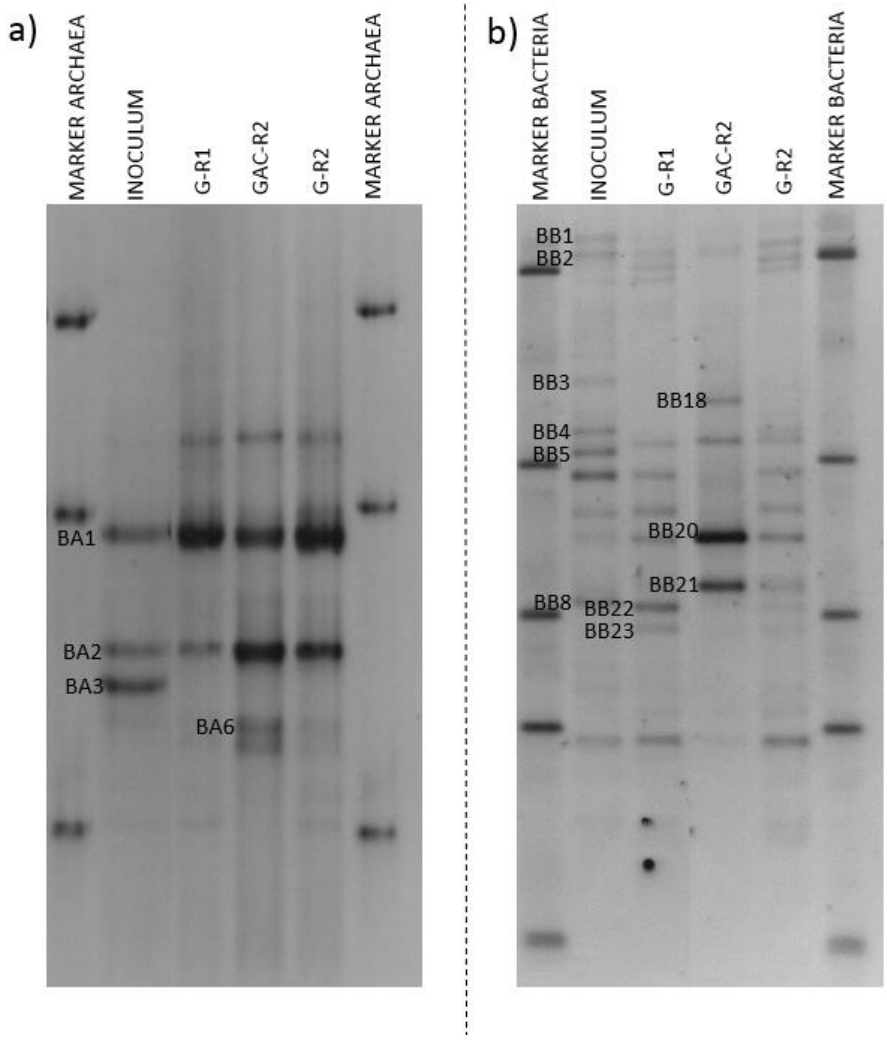


Figure 4. a) Archaeal banding pattern of the different biomass samples analysed through DGGE. b) Bacterial banding pattern of the different biomass samples analysed through DGGE. G-R1 and G-R2 labels refer to granular sludge in control and GAC-supplemented reactors, respectively; the GAC-R2 label refers to GAC-attached biomass.

Table 2. DGGE band designation, accession numbers in GenBank and levels of similarity to related organisms according to Figure 4.

DGGE band	Closest organisms in GenBank (accession number)	Similarity (%)
BA1	*Methanosaeta concilii* (NR_102903)	98
BA2	*Methanobacterium ferruginis* (NR_113045)	99
BA3	*Methanomethylovorans uponensis* (NR_133781)	99
BA6	*Methanobacterium petrolearium* (NR_113044)	99
BB1	*Anaerosporomusa subterranea* (NR_152052)	77
BB2	*Anaerosporomusa subterranea* (NR_152052)	77
BB3	*Desulfobulbus rhabdoformis* (NR_029176)	96
BB4	*Sulfuricurvum kujiense* (NR_074398)	98
BB5	*Sulfuricurvum kujiense* (NR_112144)	95
BB8	*Desulfomicrobium aestuarii* (NR_132594)	93
BB18	*Geobacter psychrophilus* (NR_043075)	95
BB20	*Geobacter argillaceus* (NR_043575)	95
BB 21	*Geobacter bremensis* (NR_026076)	93
BB22	*Geobacter uraniireducens* (NR_074940)	94
BB23	*Geobacter luticola* (NR_114303)	84

Regarding band identification, Table 2 shows the band designation, the closest microorganism found in the GenBank and their similarity. In case of archaeal DGGE, the predominant bands identified in the final samples (G-R1, G-R2 and GAC-R2). BA1 was *Methanosaeta concilii*, an acetoclastic methanogen typically found in the anaerobic granular sludge from reactors with low VFA concentrations and, one of the species more frequently identified in DIET when conductive materials have been added to anaerobic reactors.[29,30] BA2 was *Methanobacterium ferruginis*, a hydrogenotrophic methanogen, which additionally can produce methane from isopropanol,[31] so its high intensity could be attributed to the appearance of this intermediate compound (isopropanol) from the ether cleavage of E2P. BA6 was *Methanobacterium petrolearium,* another hydrogenotrophic methanogen.[31] The intensity of these two bands (BA2 and BA6) was higher in the GAC-R2 sample, in agreement with previous studies carried out in reactors that were supplemented with conductive materials, where these species have been frequently found.[32] Furthermore, this could be related to the higher degradation rate of isopropanol observed in the GAC reactor (R2). BA4 was identified as *Methanomethylovorans uponensis* a

methylotrophic methanogen.[33] This microorganism was found only in the inoculum, since no methanol was used as a substrate and it was not formed from other substrates.

In case of bacterial DGGE, significant bands identified in the inoculum were BB1 and BB2 as *Anaerosporomusa subterranea*, an anaerobic bacterium that is able to ferment short chain carboxylic acids.[34] Band BB3 was *Desulfobulbus rhabdoformis*, BB4 was *Sulfuricurvum kujiense* and BB5 was *Desulfomicrobium aestuari*. These microorganisms have a sulphur-based metabolism as sulphate-reducing bacteria,[35,36,37] which could indicate a high sulphate content in the system where the inoculum was developed. These bands (BB3, BB4, BB5 and BB8) were not detected in the final samples, as the sulphate content of the wastewater was very low. The predominant bands identified in the final samples were BB18 as *Geobacter psychrophilus*, BB20 as *Geobacter argillaceus*, BB21 as *Geobacter bremensis*, BB22 as *Geobacter uraniireducens* and BB23 as *Geobacter luticola*. All of these microorganisms belong to the *Geobacter* genus, which is the most well-known electro-donating bacteria in DIET.[32] It is also remarkable that practically every band identified in the biomass attached to the activated carbon particles (GAC-R2) was associated to these electro-active microorganisms; the same was observed for G-R1 and G-R2. However, in these samples (G-R1 and G-R2), there was a considerable number of bands detected that were not possible to identify and probably differ from the *Geobacter* genus. This could indicate that, although other species can growth in this system, *Geobacter* species are favoured by the substrate, and practically only *Geobacter* was able to grow on the activated carbon surface.

It is also important to note that none of the predominant *Geobacter* species identified in our study have been reported as able to perform DIET. In fact, only some few of *Geobacter* species i.e. *G. sulfurreducens*, *G. metallireducens*, and *G. hydrogenophilus*, are conclusively demonstrated to perform DIET, which has been usually related to their ability to synthesize conductive pili and/or to produce c-type cytochromes.[11] In this sense, abiotic conductive

material can either substitute for e-pili as electrical connection between species or enhance the effectiveness of e-pili.[10] So, DIET in the presence of GAC may not necessarily require pili and/or c-type cytochrome to form a biological electrical connection. In fact, the addition of abiotic conductive materials with specific enrichment of bacteria or consortia of methanogens not previously shown to be capable of DIET, even different from *Geobacter* species, such as *Syntrophomonas*, *Thauera*, and *Methanospirillum* species, have been shown as able of extracellular electron transfer.[38] It seems that a better understanding of DIET mechanism in presence of conductive materials is still needed.

3.2.2. High-throughput sequencing

The microbiological diversity in a sample can be represented by the alpha diversity, which can be quantified through the rarefaction curve, which also is a valuative criterion of properness for microbial analysis.

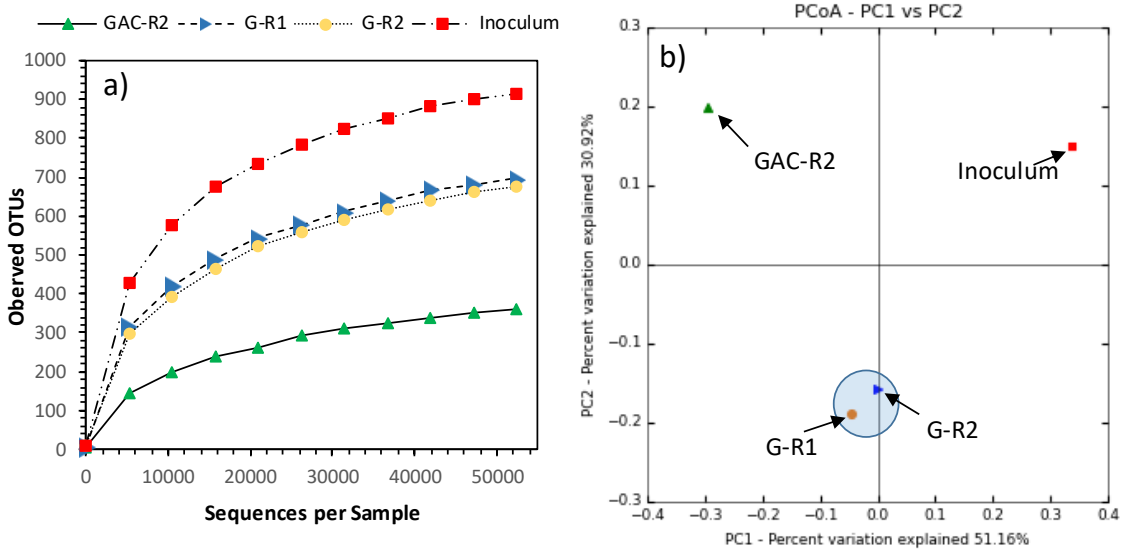
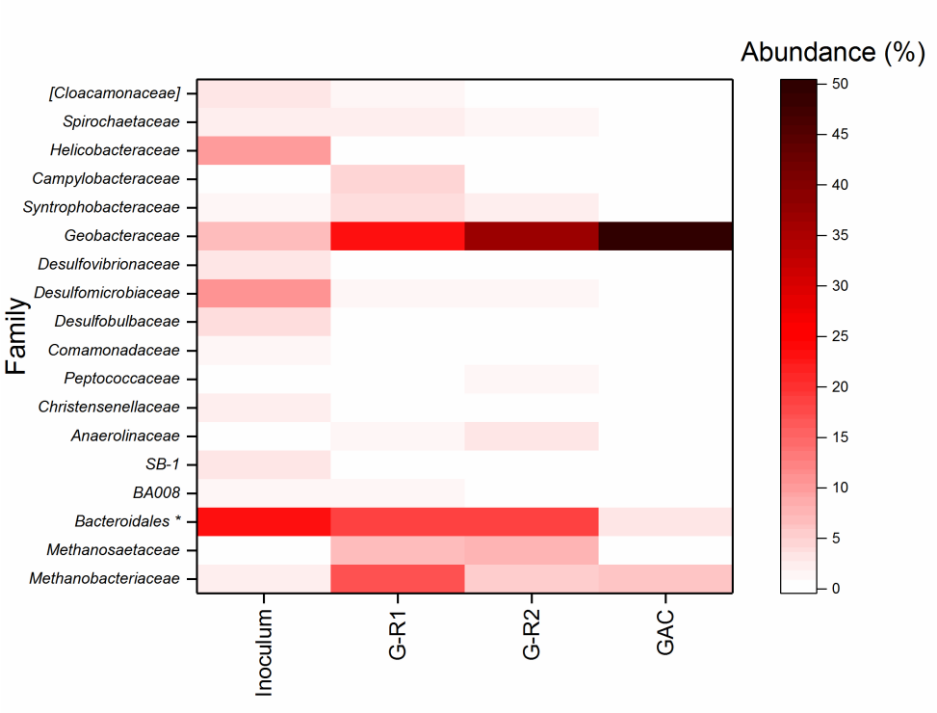


Figure 5. a) Rarefaction curve of the analysed biomass samples. b) Principal coordinate analysis of the analysed biomass samples.

Figure 5a shows the rarefaction curve of the different biomass samples taken in the experiment. The saturation of every rarefaction curve indicates good coverage of the microbial community

362 analysis. The rarefaction curve of the inoculum shows a higher (>900) number of observed
363 operational taxonomic units (OTUs) than in the granular samples from R1 and R2 (678 and 697,
364 respectively) which, in turn, are considerably larger than the number of OTUs found for the
365 biomass attached to the activated carbon. This indicates that the biodiversity was higher in the
366 inoculum than in the granules from R1 and R2 and in the biomass attached to the activated
367 carbon, as reflected in the Shannon index (6.17 for the inoculum, 5.28 for the granular biomass
368 of R1 (G-R1), 5.15 for the granular biomass of R2 (G-R2) and only 1.85 for the biomass attached
369 to the GAC (GAC-R2)). This is likely be due to the source from which the inoculum was taken, i.e.
370 a brewery wastewater treatment plant where the wastewater is known to be rich in nutrients
371 with various carbon sources; however, in R1 and R2, the carbon sources were limited to only
372 ethanol (from S1 to S3) and ethanol and E2P (from S4 to S7). Additionally, as the biomass
373 attached to the activated carbon (GAC-R2) had a lower biodiversity than the granules present in
374 the reactors (G-R1 and G-R2), indicating that only a few species were able to grow on the surface
375 of the activated carbon, or only a few species had a competitive advantage growing on it.

376 Principal coordinates analysis (PCoA) allowed us to compare the similarity of the different
377 samples; it is representative of the beta diversity, as it analyses the biodiversity between
378 different samples. Figure 5b shows the PCoA analysis for the biomass samples. The granular
379 biomass of R1 and R2 was grouped in a cluster, so the biodiversity of these samples was very
380 similar; the granular biomass in the different reactors underwent a very similar evolution from
381 the inoculum, which is depicted in the PCoA (Figure 5b) as an isolated group far from the other
382 samples. The biomass attached to the activated carbon was also isolated. This could indicate
383 that the predominant microorganisms that were able to grow on this surface were different to
384 the predominant microorganisms present in the inoculum and in the granular biomass, or it
385 could indicate that the microbial community that developed on the GAC was very specific and
386 only a few microorganisms were able to grow on it.



387

388 **Figure 6. Heatmap of the most abundant families in the samples analysed by high-throughput**

389 **sequencing.**

390 The predominant families from each sample are arranged as a heatmap in Figure 6. Regarding

391 microbial identification, a shift in the predominance of the different families was found from the

392 inoculum to the final samples (G-R1, G-R2 and GAC-R2), where some of the families such as

393 *Desulfomicrobiaceae*, *Desulfobulbaceae* and *Desulfovibrionaceae*, i.e. sulphate-reducing

394 bacteria,[39,40] were predominant in the inoculum and decreased in the rest of the samples. This

395 agrees with the DGGE results, as the predominant bands in the inoculum belonged to bacteria

396 with a metabolism based on sulphate. This change is clearly related to the change of the

397 substrate used and the operational conditions due to the low sulphate concentration in the feed

398 of R1 and R2. Conversely, some families with a low abundance in the inoculum increased notably

399 during the process, as in the case of *Anaerolinaceae*, *Syntrophobacteraceae* and especially

400 *Geobacteraceae*. The abundance of the latter family increased from 7% to 24% and to 37% in

401 the granules of R1 and R2, respectively. Therefore, the substrate and the conditions used could

402 explain the enrichment of this family, but the significant difference could be related to the

capacity of GAC to accelerate the metabolism and the growth of this microorganism, as reported by Liu *et al.*[14] Besides, the GAC surface was colonised mostly by *Geobacteraceae* family. Its abundance on the GAC was notably increased up to 84%, which suggested that GAC addition promoted the growth of electro-active microorganisms, such as the members of *Geobacteraceae*. However, bacteria that had a relatively high abundance in the granules (G-R1 and G-R2), such as *Syntrophobacteraceae* or *Bacteroidales*, did not grow on the GAC (GAC-R2), or their abundance decreased considerably. This could be related to the competitive advantage of *Geobacteraceae* to grow on the GAC surface. In this regard, Zhao *et al*.[41] reported that ethanol, as special substrate, could stimulate the methanogenic communities to enrich Geobacter and perform DIET. Regarding the methanogenic archaea developed on the GAC, no *Methanosaetaceae* were found and only *Methanobacteriaceae* were identified on the GAC with a relative abundance of 6%.

Comparing the methanogenic archaea found in the inoculum with the rest of the samples, it has to be highlighted that only the family *Methanobacteriaceae* was found in the inoculum with a relative abundance of 2%, which increased from 6% to 17% in the rest of the samples. Despite the very similar archaeal diversity of the granular biomass from R1 and R2, there were some differences. The abundance of the *Methanobacteriaceae* family was considerably higher in R1 (17%) than in R2 (6%) and the *Methanosaetaceae* family was slightly higher in R2 (10%) than in R1 (5%).

3.2.3 DGGE vs. high-throughput sequencing

The microbial community was analysed using two different molecular tools, and quite similar results were obtained by both techniques. For example, a smaller number of bands was detected in GAC-R2 than in the rest of the samples, indicating lower biodiversity, which matches with the lower Shannon index obtained from high-throughput sequencing for the sample fixed on the GAC. Additionally, the DGGE bands identified in the final samples (G-R1, G-R2 and GAC-R2)

belong to the *Geobacter* genus (*Geobacteraceae* family), although there were other bands present that were not possible to identify in G-R1 and G-R2. Similar results were obtained through high-throughput sequencing, with the relative abundances of the *Geobacteraceae* family ranging from 26% to 84%. Additionally, similar results were obtained by both techniques for the inoculum, where the predominant bands in DGGE were associated with microorganisms with a metabolism based on sulphate, and through high-throughput sequencing, the predominant families in this sample were identified as sulphate reducers. However, the two techniques also showed noticeable differences. DGGE presented some disadvantages, as it was not possible to identify some predominant bands and therefore the information about the microbial community was not complete. Additionally, DGGE is very high time-consuming and has a relatively low high throughput. In contrast, high-throughput sequencing technology provides a complete report about the microbial communities in each sample and, thanks to continuous improvement and the reduction in cost, it has become established as the most commonly used technique to analyse microbial communities in bioreactors. However, remains an expensive technique despite decreasing costs,[42] and most metagenomic reads are still unidentified due to the lack of reference genomes in this type of application.[43]

4. Conclusions

GAC supplementation substantially enhanced the response of the anaerobic system to organic load shocks and reduced the acclimation period for E2P degradation. This improvement was found to be related to an increase in the degradation rate of intermediates (acetone and isopropanol) which, in turn, was related to the effect of GAC on the microbial community. There was a decrease in the biodiversity of the microbial community from the inoculum to the final samples in the final granular biomass; biodiversity was even lower in the biomass developed on the GAC surface, which revealed microbial specialisation. This evolution was attributed to the low complexity of the wastewater, as well as to GAC addition, which promoted the growth of

specific electron donor bacteria (*Geobacter*). However, both reactors showed very similar global performance in the steady state, due to the almost identical microbial evolution of the granular sludge in both reactors. The bacteria present on the GAC surface, responsible for promoting DIET, were also found to some extent in the granules of the control reactor (R1).

5. Conflict of interest

There are no conflicts of interest to declare.

6. Acknowledgements

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Table S1 Macronutrient and micronutrient supplementation in reactor.

Compound	**Dose, mg g^{-1} COD**	**Compound**	**Dose, mg g^{-1} COD**
NH_4Cl	15.7	H_3BO_3	0.1143
Yeast extract	7.5	EDTANa	0.100
KCl	6.1	$(NH_4)_2Mo_7O_{24}\cdot 4H_2O$	0.0625
$(NH_4)_2HPO_4$	5.6	Al_2O_3	0.0595
$FeCl_3\cdot 6H_2O$	0.4208	$NiSO_4\cdot 6H_2O$	0.0447
$CoCl_2\cdot 6H_2O$	0.1615	$CuCl_2\cdot 2H_2O$	0.0134
$MnCl_2\cdot 4H_2O$	0.1441	$ZnSO_4\cdot 7H_2O$	0.132