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Water Impact Statement

We present for first time the concept **METland** that merges Microbial Electrochemical Technologies (**MET**) with constructed wet**lands.** METlands are based on electroconductive biofilters for treating urban wastewater in decentralized systems in a sustainable way with no energy cost. Our strategy was the seed for an innovative European H2020 project devoted to construct full scale applications of METlands (www.imetland.eu).

Microbial Electrochemical Systems outperform fixed-bed biofilters for cleaning-up urban wastewater

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In this work we present for first time the concept of integrating Microbial Electrochemical Technologies (MET) with the natural wastewater treatments biofilters used in constructed wetlands (CW) to form METlands. In order to validate this technology, three lab-scale horizontal subsurface flow (HSSF) biofilters, two hosting electroconductive material and one gravel biofilter (control) were operated for 525 days to define the best design and operational conditions to maximize removal of wastewater pollutants. Organic loading rates tested ranged from 2 to 24 g BOD_5 $m^{-2}d^{-1}$ at hydraulic retention times (HRT) from 4 to as low as 0.5 days, respectively. The electroconductive biofilter showed the best COD and BOD removal rates per volume of bed, achieving mean values of 213 g COD $m^{-3}d^{-1}$ and 119 g BOD m^{-3} d^{-1} at the lowest HRT (0.5 d). Ammonia and total nitrogen maximum removal efficiency at 3.4 days of HRT were 97 and 69 %, respectively, in the electroconductive biofilter. Bacterial communities were studied by 16S rDNA Illumina sequencing with the aim of understanding the role of the electrically conductive material in selecting microbial populations. *Deltaproteobacteria* (a known electroactive taxon) were enriched in presence of electrically conductive bed. *Geobacter* and *Geothrix* were the dominant genera in the deeper zone of the electrically conductive bed where oxidation of organic matter occured. The results suggest that the enhancement in biodegradation rate will significantly reduce the area requirements of classical CW.

Introduction

Conventional wastewater treatments require high energy, operation and maintenance costs. In addition, due to population growth and urban expansion, the volume of sewage sludge produced by wastewater treatment is constantly increasing [1]. Thus, a different water-energy nexus is required to cope with the future global water demand.

Since the discovery of electroactive microorganisms, Microbial Fuel Cells (MFC) were proposed to play an important role in wastewater treatment for converting the waste into clean energy, by oxidizing organic and inorganic matter to generate electrical current [2,3]. In these devices, electrons produced by the microbial metabolism are first transferred to an electrode (anode), and then to a second electrode (cathode) via a conductive material containing a resistor [3]. In this configuration, the anode act as terminal electron acceptor as any other natural acceptor like oxygen, nitrate or Fe(III). The clear advantage of exploiting electro-stimulated communities is that electrodes can boost microbial metabolism in anaerobic systems that are typically electron acceptor limited. Electroconductive material may represent an inexhaustible source of electron acceptors, hosting the additional advantage of providing a more easily modulated redox potential compared to standard, low-reducing redox species that generally drive these systems [4]. The redox potential of the anode depends on the chemistry and bioelectrochemistry around the electrode. Moreover, the electrochemical characteristics of those microbial-assisted devices can be simply controlled by altering their configuration. Thus, they can be operated in different configurations, such as i) short-circuit, no resistors between electrodes [5]; ii) MFC, able to harvest energy in presence of a resistor [6]; and iii) Microbial Electrolysis Cell (MEC) by poising a certain potential through a potentiostat or a power source [7,8].

A suitable scenario for testing this emergent technology is the Constructed Wetlands (CW) since they are a good alternative for wastewater treatment in small communities and are used worldwide [9]. Low cost operation and maintenance, low energy requirements, low production of sewage sludge (just in primary treatment) and good landscape integration are some of the most attractive advantages of CW compared to conventional treatment systems [10]. However CW treatment is constrained by limitations such as large land requirements (3-10 m^2 PE^{-1} * depending on design) (Vymazal and Kropfelova, 2008, Tilley et al., 2008) and clogging

* PE Population equivalent is the number expressing the ratio of the sum of the BOD load produced during 24 hours by industrial facilities and services to the individual BOD load in household sewage produced by one person in the same time. For practical calculations it is assumed that one unit equals to 60 g of BOD per 24 hours.

by the accumulation of solids [13,14]. Recommended surface organic inlet load for HSSF CW is reported as 6.0 g BOD_5 m^{-2} d^{-1} in order to achieve a value under 30 mg BOD_5 L^{-1} in the effluent and avoid clogging [15,16]. HSSF CW were initially presented as environments that could take advantage of depth-depending redox potential gradients [17,18]. Previous reports argued that redox conditions in CW could be controlled by altering the organic loading rate, the hydraulic design and the mode of operation [19]. Following this strategy, different groups have integrated MFC elements to lab-scale CW with the purpose of harvesting electricity [20–22]. In spite of using wastewater as organic fuel, the power densities reported were as low as 1.84 – 44.63 mW m^{-2} [23], which is a range typical for sMFC operating in soil or sediments, but still far from 10 W m^{-2} values obtained using filter press bioelectrochemical reactors [8]. This is mainly due to the fact that redox gradients are not broad enough in this kind of environments and *in situ* implementation of power-harvesting devices is indeed limited.

However, we still believe that CW are a suitable environment for implementing microbial electrochemical systems. Our aim was not to harvest energy but to enhance the rate of pollutant removal by converting the classical inert biofilter into an electroconductive biofilter where its redox state could be tuned or controlled by electrochemical tools. Our results revealed how the integration of METs in wetlands resulted in a powerful hybrid technology so-called METland [24], that strongly outperforms the treatment of urban wastewater through the stimulation of different microbial populations.

Experimental

Design and construction of electroconductive biofilters

In this study, four laboratory-scale HSSF biofilters were constructed for determining the best design and operational conditions to maximize wastewater pollutants removal. A control unit used standard siliceous gravel (Ø 6-12 mm) as biofiltering bed (Fig.1, A). An electroconductive bed configuration (Fig.1, B) was constructed with a single material, acting as a whole electrode. This configuration did not allow the conversion of microbial metabolism into electrical current to be monitored, since anode and cathode were not differentiated. In order to harvest electrochemical information about the process, a three electrodes system was additionally constructed by using a hybrid unit made of inert gravel and polarized coke bed (Fig.1, C). An additional hybrid unit operating under short-circuit (Fig.1, D) was constructed as control. In these hybrid biofilters, conductive material was vertically inserted into the gravel. The short-circuit hybrid unit acted as a single electrode without differentiated anode and cathode.

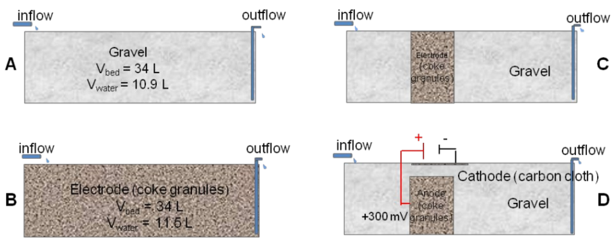

Fig.1 Simplified design of the four systems A) Gravel biofilter (control), B) Coke biofilter, C) Hybrid biofilter, D) Hybrid polarized biofilter.

The conductive material in the bed was coke granules (Ø 5-10 mm). The dimension of the biofilters were 0.52 m long, 0.34 m wide and 0.30 m high, and material layer was 0.20 m deep, with a total bed volume of 0.034 m^3 and a water volume of 0.011 m^3. Each biofilter had a drainage pipe, located on the flat bottom, for effluent discharge and the water level was kept below the surface.

The hybrid polarized biofilter hosted a coke anode of 0.006 m^3 as schemed in Fig.1. A plate of graphite (3 cm x 3 cm x 0.5 cm, Sofacel) buried into the coke anode acted as electron collector. The cathode was made of carbon cloth (0.34 m x 0.15 m, Resinas Castro, 420 g m^{-2}). Anode and cathode were connected by a copper wire to a potentiostat unit (Nanoelectra S.L., Spain). A third electrode (Ag/AgCl) buried in the anodic bed acted as reference to polarize the anode at 0.3 V (vs. Ag/AgCl). The anode potential and the current were periodically measured using a digital multimeter (Model 2700, Keithley Instruments, USA). Data was recorded every 10 s on a spreadsheet using ExceLINX_(Keithley) via an interface card (GPIB Interface Boards, Keithley) linked to a personal computer. The performance of the polarized biofilter was evaluated in terms of coulombic efficiency (CE, %) comparing the total electrons harvested by the anode to the electrons possibly generated by the microbial oxidation of the substrate. For continuous flow through the system, we calculate CE based on the COD change, and the flow rate, q [25], as

$$CE = \frac{8\,I}{Fq\,\Delta COD}$$

where 8 is a constant used for COD, based on conversion from gO_2 (MW = 32 g mol^{-1}) to mol e^- (4 mol e^-/mol O_2), I is the current obtained over time and F is the Faraday's constant.

The systems were operated in parallel and fed with real urban wastewater from the municipality of Carrión de los Céspedes (Sevilla, Spain) (2500 inhabitants) under discontinuous flow regime during 525 days (75 weeks). Wastewater was pretreated in an Imhoff tank in order to remove solids and prevent a potential clogging of the systems. The feeding from the Imhoff was made by programmed pumping, by means of 12 daily periods, simulating the production of wastewater in small populations [26]. Several organic loading rates were tested (2.0 ± 1.0; 4.2 ± 0.7, 9.2 ± 2.8, 13.8 ± 9.5 and 24.0 ± 12.7 g BOD_5 m^{-2} d^{-1} in average) at the following hydraulic retention times (HRT): 4.0, 3.4, 1.7, 0.8 and 0.5 days, respectively.

Physical, chemical and statistical analysis

BOD_5, total suspended solids (TSS), total nitrogen (TN), ammonia (NH_4) and nitrate (NO_3) were analysed weekly; COD was analysed twice a week, following the standard methods (APHA-AWWA-WEF, 2005). Temperature (T), pH, electrical conductivity (EC), dissolved oxygen (DO), and redox potential (ORP) were measured weekly with a handheld multiparameter (YSI 556 MPS). Samples were taken at the inlet and the outlet of the systems and water flow was daily measured. Moreover, hybrid systems were also sampled through sampling tubes buried in the bed, before and after the electroconductive barrier (anode), in order to calculate the coulombic efficiency. Inlet wastewater analyses is shown in Table 1S. Removal rates were calculated as grams per cubic meter of bed material per day. Removal efficiencies were calculated as percentage.

Statistical procedures to evaluate the effect of HRT for every water quality parameter were conducted using the Statgraphics Centurion XVII statistical software package. T-test or Wilcoxon tests were used to determine the differences of every water quality parameter among the effluents, depending on the type of data (95 % confidence).

Microbial communities

Sampling, DNA extraction and 16S rDNA sequencing. Samples were taken from lab-scale biofilters and inlet wastewater to determine the composition of their microbial community at four different spots: anode in the hybrid polarized biofilter (B1), upper area of the coke biofilter (B5), upper area of the gravel biofilter (B6) and inlet wastewater (B7). Either granules of coke (B1, B5) or gravel pebbles (B6) were sampled with tweezers and loosely attached bacteria were removed by dipping them in 3 consecutive sterile saline solutions (50ml, NaCl 7 g/l). Coke and gravel pebbles were then frozen for 1 week until performing DNA extraction. Around 10 granules/pebbles were extracted per spot. DNA was extracted with PowerSoil spin columns (MO BIO Laboratories), suspended in 60 µl of sterile MilliQ water and quantified with PicoGreen (Invitrogen). A total of 3 ng of DNA were amplified with primers 515F-CS1 (<u>ACACTGACGACATGGTTCTACA</u>*GTGCCA GCMGCCGCGGTAA*) and 806R-CS2 (<u>TACGGTAGCAGAGACTTGG TCT</u>*GGACTACHVGGGTWTCTAAT*). The polymerase used was Q5 Hot Start High-Fidelity (New England Biolabs) and the PCR conditions were: initial denaturation at 98°C for 30'' followed by 30 cycles of 98°C x 10'', 60°C x 20'' and 72°C x 20'', and a final elongation step of 72°C for 2'. A 1/100 dilutions of PCR products were then re-amplified (15 cycles) with Illumina´s primers. Finally, products were run on a Bioanalyzer (Agilent) and the successful generation of equimolar pools was confirmed by qPCR. Sequencing was performed in a MiSeq equipment using the 2x250 bp format and following Illumina's protocol.

The Illumina Miseq sequence reads have been deposited in the European Nucleotide Archive (ENA) database under accession Nr. PRJEB10685.

Bioinformatic analysis. The total sequence reads were analysed with the QIIME 1.7 pipeline [28] with few stitches along the way. Briefly, complementary reads were merged using fastq-join [29]. Subsequently, our quality filtering strategy removed complemented sequences that had one of the following characteristics: (i) deviated more than 10 bp from the expected length (292); (ii) contained primers with more than 1 mismatch; or (iii) contained nucleotides with Phred score <20. Filtered seqs were organised in OTUs by de novo picking using Usearch [30] and one representative sequence per OTU was chosen. Taxonomy was assigned using the GreenGenes database [31] version 10_12 at the 97% identity rate. Furthermore, sequences were aligned and a tree generated using FastTree 2.1.3 [32]. Finally, in order to investigate alpha diversity with QIIME, OTUs containing less than 0.005% of the total sample reads were removed according to Bokulich [33]. The results have been represented as relative abundance of a specific sequence in every sample. Taking into account the possible effect of deviation introduced by the implemented protocol and that not all the bacterial species have the same number of copies of 16S rRNA gen in their genomes [34], the values can be related to percentage of cells of every species that were part of the sampled communities.

Results and discussion

HSSF CW are biofilter setups that exploit biofilm-based natural process by means of inert material like gravel with the purpose of treating urban wastewater. Plants are typically integrated in CW for oxygenating the root zone and for providing aerobic microorganisms an habitat within the anoxic environment [35]. Our approach consists in substituting inert material for an electroconductive material in order to stimulate electroactive microorganism and consequently biodegradation rates. Due to the oxygen supply role by plants we did not include vegetal species in our experimental set up in order to achieve a better control of the redox interaction between bacteria and bed.

Urban wastewater treatment by horizontal subsurface flow (HSSF) biofilters: electroconductive versus non-electroconductive biofilters

Influence of the material on the wastewater pollutants removal. In order to quantify the influence of the material, we tested two independent HSSF biofilters fully constructed with electroconductive and inert material (Fig.1A,B). The organic matter removal rates, in terms of COD and BOD_5, were similar under a low organic loading rate regardless of the material (Table 2S). However, significant differences among both systems appeared when the organic loading rate was increased. The coke biofilter performed removal efficiencies close to 100% despite increasing the organic loading rate, while the gravel biofilter efficiency decreased as the organic loading rate increased (Fig.2). Indeed, the coke biofilter showed the best COD and BOD_5 removal rates, achieving mean values of 213 g COD $m^{-3}d^{-1}$ and 119 g BOD_5 m^{-3} d^{-1} (Table 2S). Furthermore, the gravel biofilter showed a more variable performance.

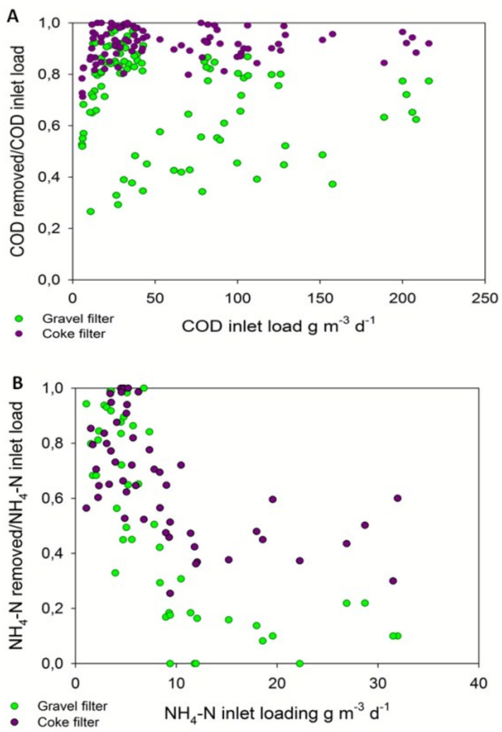

Fig.2 A) Relation between normalized COD removed and COD inlet loading of the coke and the gravel biofilters, B) Relation between normalized NH_4-N removed and NH_4-N inlet loading of the coke and the gravel biofilters.

Statistical tests revealed that there were significant differences ($p < 0.05$) in the effluent's concentration of COD and BOD_5 at every HRT (Table 3S) when the coke and gravel biofilters where compared. The coke biofilter biodegradation rates led to effluents with residual values up to 3-fold lower for COD and 4.5-fold lower for BOD_5 (Fig. 3). COD and BOD_5 coke biofilter effluent values never exceeded the limits of discharge, which are 125 mg COD L^{-1} (or > 75 % removal) and 25 mg BOD_5 L^{-1} (or 70-90 % removal) (Dir. 91/271/EEC of 21 May 1991)[36], in contrast with the gravel biofilter performance from 3.4 days of HRT onwards, which average effluent concentration exceeded 25 mg BOD_5 L^{-1} (Fig. 3). Even at the lowest HRT the performance of the coke biofilter fulfilled the COD and BOD_5 discharge requirements in percentage (91 % and 96 %, respectively), compared to hardly 73% and 86 % for the gravel biofilter (Table 2S). Caselles-Osorio and García [37] reported COD removal efficiencies of 71-85 % in intermittent fed HSSF CW experimental systems with a nominal HRT of 3.4 days, which is comparable to removal efficiencies of our control system at the same HRT (83 %). Coke biofilter achieved mean BOD_5 removal rates as high as 99 % at high HRT (3.4 days).

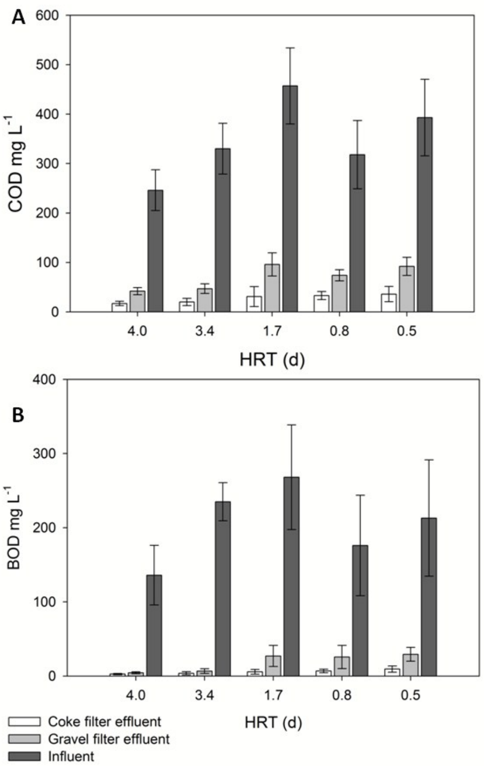

Fig.3 COD (A) and BOD_5 (B) influent and effluent average values of the coke and gravel filters. Error bars represent 95 % confidence interval.

The BOD_5 surface inlet loads applied at 1.7, 0.8 and 0.5 days of HRT (Table 2S) were 1.5, 2.3 and 4-fold, respectively, the recommended load (6.0 g BOD_5 $m^{-2}d^{-1}$) and BOD_5 average values of the coke biofilter effluent were always under 10 mg L^{-1} (Fig. 3). Even at very high inlet organic loads, the coke biofilter had a great capacity to remove organic matter, without any evidence of clogging during the long course (525 days) of the experiment. A remarkable conclusion is that just the coke biofilter fulfilled the Directive for COD and BOD_5 at a HRT as low as 0.5 days. In contrast, for standard gravel biofilter a HRT as high as 3.5 days was required for fulfilling the limits. Moreover, there were not significant TSS differences in the effluents of the two biofilters, and both fulfilled the limit values of discharge (35 mg L^{-1}) (Table 1S).

Nitrogen removal was also analysed under both electroconductive and inert materials and a very similar result was found. Statistical analysis revealed significant differences ($p < 0.05$) among TN and NH_4-N effluent concentrations at every HRT. The coke biofilter exhibited the highest removal rates at every HRT (Table 4S). Interestingly, differences with gravel biofilter were more noticeable than those found for organic matter removal. In the coke biofilter, the maximum amount of nitrogen was removed at 0.5 days of HRT (TN 11.9 gN $m^{-3}d^{-1}$; NH_4 11.2 gN $m^{-3}d^{-1}$) although the removal efficiency (%) decreased with decreasing HRT. This trend has been reported in other studies [16,38]. The coke biofilter showed maximum average removal efficiency values at 3.4 days, 97% of ammonia and 69% of total nitrogen compared to 71% and 51%, respectively, in the gravel biofilter. The minimum values were reached at 0.5 days, 39 % of NH_4-N and 37 % of TN compared to 16 % and 19 %, respectively, in the gravel biofilter (Table 4S). Fig. 2B shows that the coke biofilter had a trend to maintain higher removal rates than gravel biofilter. The higher biodegradation rates generated effluents with residual TN and NH_4-N significantly lower (Fig. 1S). The results demonstrate that the coke biofilter removed at least 2-fold the amount of TN and 2.5-fold the amount of NH_4 than removed by the gravel biofilter (HRT 0.5 days). Therefore, at HRT shorter than 4 days nitrification was higher in the coke biofilter compared to the gravel biofilter. Moreover, at lower HRT, ammonia concentration in the effluent increased while nitrate was decreased (Fig.1S). The improvement of the conversion of ammonia to nitrate and nitrogen removal suggests the enhancement of other metabolic pathways in the electroconductive bed.

Electrochemical analysis using hybrid electroconductive setups. In order to quantify the role of the electroconductive bed for accepting charge from microbial metabolism we constructed a hybrid polarized biofilter (figure 1D). In contrast with the sole-coke biofilter, this setup allows an accurate control of the electrical current by polarizing the system at 0.3 V (vs. Ag/AgCl).

The electrical current monitored throughout the assay revealed an expected profile, a stable value around 100 mA was measured (Fig. 4). Interestingly, an increase in the organic loading rate did not result in a clear increase in electrical current, suggesting that the electroactive biofilm was not limited in electron donor. In contrast, the increase in the organic loading rates showed very good correlation with the organic removal rates only in the presence of electroconductive material so we concluded that some other biodegradation pathways, although not contributing to current production, are definitively being enhanced. As the electron donor is not a limiting factor, other degradation routes must have a major influence on the performance. In that sense, coulombic efficiency (CE) ranged from 37 % at low organic loading rate to 9 % at maximum organic loading rate, which indicates that low organic loading rates enhance the CE. The bacteria can biodegrade part of the COD through fermentation or the use of alternative electron acceptors [39] such sulphate or nitrate. This is consistent with previous reports that showed how, under higher organic loading rates, electron flow is channelled towards methanogenesis or sulphate reduction so CE is reduced [39]. Methane emissions are common in HSSF CW because these systems present appropriate environmental conditions for methanogens and sulphate-reducing bacteria. These Archaea and Eubacteria require environments with similar redox potentials and use the same types of electron donors (i.e., hydrogen, methanol, and acetic acid) [9]. Methane emission rates are very variable and they are usually greater at the inlet than the outlet, given that methanogens activity is directly dependent of the organic load [40]. Further research about this topic should be carried out to evaluate the contribution of METlands to methane emissions.

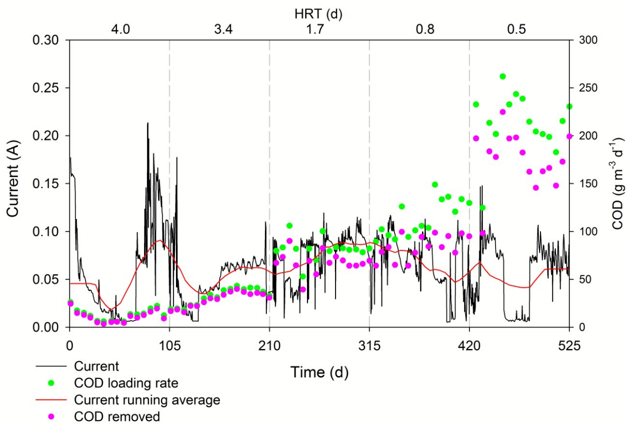

Fig.4. Profile of electrical current, COD loading rate (g $m^3 d^{-1}$) and COD removal rate (g $m^3 d^{-1}$) during long term operation of the hybrid biofilter polarized at 0.3 V (vs. Ag/AgCl).

Together with the hybrid polarized system, a non-polarized hybrid biofilter was also constructed (Fig. 1C) to evaluate the influence of the polarization versus the mere effect of the coke. Interestingly, despite polarizing the anode our assays did not reveal significant differences ($p>0.05$) in terms of COD and BOD removal among the two hybrid configurations (Fig. 2S). This fact strongly suggests that the electroconductivity of the material exert a positive influence on the microbial metabolism regardless of the existence of an electron flow among the different electrodes. Our Hybrid biofilter is a single electrode configuration, a simplified design of a short-circuited system that cannot provide current but optimizes the pollutants removal. In that sense, our results are consistent with previous studies that reported how compact short-circuited system provided higher biodegradation performance than MFCs operating at maximum power [41].

Redox potential was measured in both the electroconductive and the gravel biofilters. There was a noticeable redox potential gradient with depth and distance from the inlet in the systems which corresponded to COD and BOD. This gradient was greater in the electroconductive biofilter (Fig. 3S). This gradient suggests the presence of an electron flow from the deep bed to the more oxidized top layer of the coke bed.

In the hybrid systems the differences between materials were also remarkable. COD removal rates in the electroconductive bed (Table 1) were ca. 5-fold higher than in the gravel bed of the same hybrid device. Regarding nitrogen removal, both hybrid systems removed similar amounts of total nitrogen and ammonia at high and medium HRT (Table 4S).

Table 1 Urban wastewater treated by hybrid biofilter setups. COD overall averages ± SD, at HRT = 3.4 d. Removal efficiencies in the conductive bed (%) were referred to the COD before conductive bed.

COD levels (mg L^{-1})	Hybrid biofilter	Hybrid polarized biofilter
Influent	231 ± 58	231 ± 58
Before conductive bed	188 ± 55	182 ± 59
After conductive bed	89 ± 49	78 ± 31
Effluent	37 ± 20	35 ± 14
COD removal		
Removed in conductive bed (g $m^{-3}d^{-1}$)	50.9 ± 24.8	55.5 ± 26.0
Removal efficiency in conductive bed (%)	52 ± 18	56 ± 14
Removed in gravel before conductive bed (g $m^{-3}d^{-1}$)	12.8 ± 7.8	15.8 ± 13.4
Removed in gravel after conductive bed (g $m^{-3}d^{-1}$)	10.4 ± 7.0	8.1 ± 4.8

Microbial communities

The analysis of four microbial communities revealed 696,288 raw reads that yielded a total of 689,911 high quality sequences with an average length of 292 bp (Table 5S). This volume of sequences is around one order of magnitude greater than previously reported studies of diversity in bioelectrochemical systems [42], as result of improved sequencing technologies. Clustering these sequences generated 16,582 OTUs evenly distributed between the four samples. 2.33% of the sequence reads were not classified.

The classifiable sequences included members of 48 phyla of which an average of 64 % were *Proteobacteria,* ranging between 52% (anode of the hybrid polarized biofilter) and 74% (gravel biofilter).

Rarefaction curves showed saturation, indicating that a reasonable number of sequence reads per sample were collected to reveal diversity at the sites (Fig. 4S). Rarefaction curves indicate that predicted diversity was much less in the inlet wastewater than in the rest of the niches (around 70% of the number of identified taxa). Diversity estimators such as observed OTUs, Chao1 and Shannon-Wienner, were significantly higher for coke granules samples when compared to the gravel samples (Table 5S). The Good's coverage estimator denoted that the sizes of the libraries were enough to cover almost 100% of the bacterial communities. Shannon diversity indexes (*H*), which includes the information of both richness (the number of species present) and evenness (how the abundance of each species is distributed) were obtained for our system. They were distinctly higher (between 6.27 and 7.38) than those in other studies on electrochemical CW treating urban wastewater (4.36-5.5 [43], 5.6 – 6.3 [44]) and similar to the results of Lu et *al.* [45] (*H*: 7.33-7.47). These results, together with the high number of taxa found in the samples, indicated a very high diversity.

Weighted Fast UniFrac analysis and Correspondence analysis (CA) were used to identify the differences of the bacterial community structures based on their phylogenetic lineages. CA showed that the four communities separated distinctly from one another despite the same origin (Fig. 5S). CA plot revealed that coke and hybrid polarized biofilters are closely related and that electroactive bacteria (*Deltaproteobacteria*) had the higher component weight in both systems. Another closely related taxa to these biofilters were the classes *Holophagae* (with the genus *Geothrix*, an electroactive bacteria of the phylum *Acidobacteria*), and *Brocadiae* (phylum *Planctomycetes*). The class *Brocadiae*, involved in annamox processes, only appeared in the anode of the polarized biofilter (table 6S). *Alpha*, *Beta* and *Gammaproteobacteria* had the higher component weight in the inlet wastewater and the gravel biofilter.

Presence of *Deltaproteobacteria* as indicator of microbial electroactivity. Our analysis of microbial communities revealed the presence of similar taxonomic groups with the exception of some remarkable ones. An interesting finding was the high presence of *Deltaproteobacteria* (Fig. 5) when the electroconductive material was the substrate (27.2 % in the coke biofilter and 23.4% in the hybrid polarized biofilter) in comparison with the gravel biofilter (8.1 %). Bacteria belonging to this group have been reported associated to electroactive biofilm from the very beginning [46] as they share the capacity for generating ATP from very low thermodynamic value reactions [47,48]. In the anaerobic treatment of wastewater, *Deltaproteobacteria* assures the removal of fatty acids of low energetic value as acetate which is typically the metabolic bottleneck of these systems [49]. In addition, *Deltaproteobacteria* can compete with methanogenic microorganisms and their preponderance may reduce methane emissions. However, we cannot confirm any outcompeting effect on methanogenic populations because, apparently, some of the taxa were not amplified with the primer sets 515F/806R utilised for the sequencing [50]. In fact, only 0.1 % of OTUs correspond to the Kingdom *Archaea*, which contains the main methanogenic groups. It must also be noted that community members with multiple 16S copies may be over-represented. Nevertheless, our main purpose was to estimate those genera associated with degradation processes and electroactive bacteria, groups that were adequately represented.

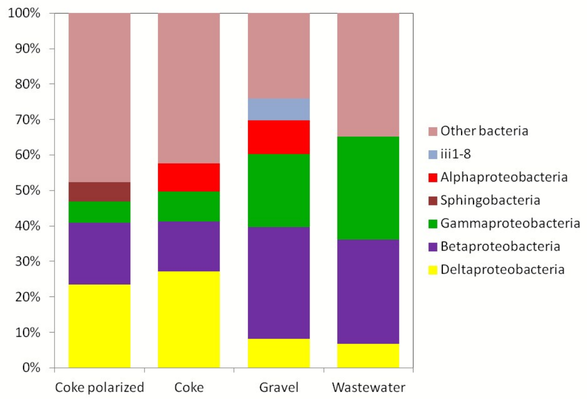

Fig.5 Relative abundances of OTUs at class level (larger than 5% in average).

Some *Deltaproteobacteria*, like bacteria from the genus *Geobacter,* are able to transfer electrons to conductive materials [51]. Indeed, the largest presence of *Geobacter* was found in the coke biofilter (2.9%) (Table 7S). Surprisingly, although at lower levels it was also found in the inlet wastewater (0.45 %) and in the gravel biofilter (0.3%). Some studies have previously reported the presence of *Geobacter* species in anaerobic digesters suggesting a role in performing direct interspecies electron transfer (DIET)[52–55] with a direct impact on methane production. Interestingly, inlet wastewater for our assays was generated in an Imhoff tank, which host environmental conditions similar to those found in an anaerobic digester. It seems reasonable to expect the presence of *Geobacter* associated with other biofilm species in our gravel biofilter. In the *Deltaproteobacteria*, it is remarkable the dominance of some genera of the family *Desulfobulbaceae* (Table 7S) in both the anode of the hybrid polarized biofilter (20.8%) and also in the coke biofilter (16.8%), in contrast with low presence in the gravel biofilter (1.6%). Moreover, other studies also reported *Desulfobulbus* species colonizing anodes [44,56–58] and, for instance, *D. propionicus* was previously reported to use the electrode surface as an electron acceptor when pyruvate, lactate, propionate or hydrogen was provided as electron donor [59]. The presence of *Desulfobulbus* is especially relevant due to its fascinating capacity for generating electrically conductive-microbial filaments [60,61]. These microbial filaments transport electrons from the bottom of sediment, rich in hydrogen sulphide, up to the oxygen-rich sediment that is in contact with the water. Interestingly, this situation is similar to the one found in our METlands where a redox gradient is generated among bottom and upper layers of the electroconductive bed. So, our results have revealed that specific microbial consortia previously related to electrical current production were selected for by our electroconductive biofilters from our inlet wastewater.

On top of that, other electroactive microorganism like *Geothrix*, an *Acidobacteria* [62], were also found in all the systems (table 7S), with a significant presence in the anode of the hybrid polarized biofilter (3.2 %) and in the coke biofilter (2.2 %). Interestingly, *Geothrix* was almost absent in the inlet wastewater and scarce in the gravel biofilter (0.2%).

Nitrogen cycle bacteria: nitrification and denitrification. Nitrogen removal is typically poorly achieved under anaerobic conditions, showing a bottleneck in the ammonium oxidizing process. Apparently this is not the case when electroconductive material is supporting the biofilm growth (Fig. 1S) since this material outperforms gravel to remove nitrogen by 2-fold (table 4S).

A deep analysis into the microbial communities' distribution may helps us to understand what different nitrogen metabolisms are active in our systems. The detection of ammonium oxidizers, like *Nitrosomonadaceae*, associated to the electroconductive material is remarkable if we consider that this family was absent in both the gravel and the inlet wastewater. Even more interesting was the presence of bacteria from the *Brocadiaceae* family (1.7%) in the anode of the polarized biofilter. This family of bacteria include several genera involved in the anaerobic oxidation of ammonia to dinitrogen via ANNAMOX [63].

Another nitrogen pathway that could be enhanced by the presence of the electroconductive material is based on direct interspecies electron transfer [64]. Focusing on nitrogen removal, it has been reported that *Geobacter* bacteria can transfer electrons directly to *Thiobacillus* which in turn may reduce nitrate [65]. Interestingly, both microbial genera are colonizing our electroconductive biofilters although further research is required to find out if these redox syntrophic relationships are the ones after nitrogen removal in our systems.

Conclusions

Problems with wastewater treatment in small communities are different that in large cities owing to the scarcity of economical and technical resources. It is necessary to find solutions that generate minimum energy cost, simple maintenance and functional robustness. With this aim, the successful integration of microbial electrochemical technologies into well tested treatments, such as constructed wetlands, represents a substantial advance since the new system can be operated a surface inlet load 4-fold higher than the standard systems. Indeed, our lab scale METland design for treating urban wastewater was able to fulfil the Directive 91/271/EEC and produced water with BOD_5 levels as low as 6 mg/L. Our research suggests that surface area requirements of classical Constructed Wetlands (CW) can be significantly reduced.

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Table of contents

Water impact: MET biofilters outperform gravel biofilters for wastewater treatment and will reduce the surface for CW, selecting certain genera of bacteria reported to be electroactive.

GRAPHICAL ABSTRACT