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Denitrifying bio-cathodes developed from constructed wetland sediments exhibit electroactive nitrate reducing biofilms dominated by the genera *Azoarcus* and *Pontibacter*

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Abstract

To limit the nitrate contamination of ground and surface water, stimulation of denitrification by electrochemical approach is an innovative way to be explored. Two nitrate reducing bio-cathodes were developed under constant polarization (-0.5 V vs SCE) using sediments and water from a constructed wetland (Rampillon, Seine-et-Marne, France). The bio-cathodes responded to nitrate addition on chronoamperometry through an increase of the reductive current. The denitrification efficiency of the pilots increased by 47 % compared to the negative controls without electrodes after polarization. 16S rRNA gene sequencing of the biofilms and sediments evidenced the significant and discriminating presence of the *Azoarcus* and *Pontibacter* genera in the biofilms from biocathodes active for nitrate reduction. Our study shows the possibility to promote the development of efficient *Azoarcus*-dominated biocathodes from freshwater sediment to enhance nitrate removal from surface waters.

Keywords : Electroactive biofilms, nitrate, 16S rRNA gene sequencing, *Azoarcus*, *Pontibacter*, bioelectrochemical systems

1. Introduction

Nitrate pollution of water arising mainly from intensive agriculture is a major environmental and health concern. The maximum acceptable nitrate concentrations in drinking waters is 50 mg.L⁻¹ of NO₃⁻ or 11.3 mg.L⁻¹ of N-NO₃⁻ [1] in Europe and only 10 mg.L⁻¹ of NO₃⁻ or 2.2 mg.L⁻¹ of N-NO₃⁻ in the United States of America [2]. To maintain drinking water below these nitrate concentration limits, polluted water has to be treated through complex and costly biological or chemical processes [3,4]. It is possible to address the issue of natural aquifers nitrate pollution by the upstream use of artificial wetlands intercepting runoff water from fertilized fields and performing depollution by microbial communities and plants before the water goes underground, for example through sinkholes infiltration [5]. However, the massive use of nitrogen-based fertilizers in developed societies causes an ever-increasing nitrate pollution of the wetlands collecting waters from the fields, limiting and saturating the nitrogen uptake capacity by plants and the denitrification capacity of plants and bacteria naturally present in the ecosystem. This in turn provokes nitrate pollution of underground waters by the release of only partially treated water into the aquifers.

Microbial electrochemical systems have been recently proposed to contribute to the stimulation and speed up of the natural denitrification processes at work in designed artificial wetlands [6]. Most microbial electrochemical systems exploit the ability of some micro-organisms to transfer electrons derived from carbon matter catabolism to a solid surface like a metal oxide or an anode, as the terminal electron acceptor for cell respiration [7,8]. Other electro-active bacterial species can, conversely, receive electrons from a solid surface and use the gained electron from the solid surface to reduce a molecule like oxygen for example. Importantly, some of these electrochemically active cathodic bacteria are able to reduce nitrate. The first example of a denitrifying bio-cathode was reported by Clauwaert *et al.* [9] and since then the number of experiments and studies on this topic has increased consistently [10–12].

To date, there are however few convincing field applications of bioelectrochemical systems that remain mostly confined to small size laboratory demonstrators. This is mainly due to the difficult scale-up of such systems (displaying high ohmic drop for example) and to the amount and cost of materials (membranes, electrodes, wires, etc.). To overcome these bottlenecks, one simple and potentially low cost device is the short-circuited microbial fuel cell also known as the microbial electrochemical snorkel [13].

In this configuration the same electrode at its rest potential has a microbial anodic side oxidizing organic matter and a microbial cathodic side reducing an inorganic substrate. A microbial snorkel would therefore be an appealing approach to explore in order to stimulate denitrification in constructed wetlands with a bioelectrochemical system consisting in the partial burying of electrodes into the sediment rich in organic matter.

In this context we investigate in this preliminary work the potential of sediments from the artificial wetland of Rampillon (Seine-et-Marne, France) to provide cathodic denitrifying biofilms at an electrode polarized at -0.5 V vs. SCE and characterize the biofilms and sediment microbial fauna with 16S rRNA gene sequencing.

2. Materials and methods

2.1. *Electrode design*

The electrodes were built following a specific design defining three internal compartments (Figure 1, Left). Stainless steel grid (Alloy 316 with 16 to 18 % of chromium, 37 % of open surface, 0.38 mm size of pore, 0.25 mm wire diameter, purchased from GoodFellowUSA®, ref:FE248710) was used to make a cylindrical structure of 15 cm height and 1 cm of diameter. Inside the stainless-steel tube, two plastic layers were fitted to obtain three separated compartments of 5 cm height (Figure 1), where 2 g of graphite granules (irregular diameter between 0.1 cm and 0.3 cm) were placed. The plastic separators between compartments were pierced with a dozen of 0.1 cm diameter holes to allow circulation of water and bacteria but not the granules from one compartment to another. A stainless-steel wire was then inserted in the electrode with direct contact with the stainless-steel grid main structure and graphite granules in each of the three compartments. Resistance measurements were carried out between different parts of the electrodes and never exceeded 10 Ω . Three of those compartmented electrodes were built for the experiment.

2.2. *Pilot design*

Six cylindrical (7.5 cm diameter) reactors were built out of PolyVinyl Chloride (PVC) opaque plastic material. In those six pilots, 500 g of crushed dry sediments collected from the constructed

wetland of Rampillon, Seine-et-Marne, France [14] were mixed with 600 mL of water collected from the same wetland. 1 g.L⁻¹ sodium carbonate (Na₂CO₃) was added in the water. The concentration of nitrate and nitrite before the experiment was negligible (below 1 mg.L⁻¹ for both NO₃⁻ and NO₂⁻). The top of the pilots was not covered to let light enter from above in order to mimic the natural conditions in wetlands. In three of the six pilots, the working compartmented electrode was partially buried in the sediments with the first compartment fully buried in the sediments, the second one at the sediment/water interface (with all the graphite granules buried) and the third one fully in water (Figure 1). A platinum wire placed in a glass tube containing 0.1 M of KH₂PO₄ and K₂HPO₄ phosphate buffer and ended by a Proton Exchange Membrane (PEM) was used as counter electrode. The reference electrode was a Saturated Calomel Electrode (SCE) obtained from SI Analytics® (E = +0.244 V vs. SHE at 25 °C). In addition to the three pilots equipped in a three electrode configuration linked to a potentiostat, three other pilots were left with only sediments and water (also supplemented with the same concentration of sodium carbonate) to be used as controls.

Here Fig. 1

2.3. *Electrochemical study*

The three designed electrodes were first left without external applied potential for four days before being polarized at -0.5 V vs SCE using a VMP3 multi-channel potentiostat (Biologic®) and the current was recorded with chronoamperometry using the EC-lab software (Biologic®). The applied potential was chosen according to previous studies showing that nitrate reduction catalyzed by electroactive bacteria occurs around -0.5 V vs. SCE [15–18].

2.4. *Pilots incubation and experiment timeline*

The six pilots, numbered 1, 2 and 3 and fitted with electrodes (Figure 1, Right) and numbered 4, 5 and 6 without electrodes and serving as controls were all placed at constant temperature (23 °C), with 9 hours of artificial light per day for 5 days a week for 26 days (623 h). The pH was measured every two days and remained between pH 6 and pH 8. Water samples for chemical analysis were also taken right before and after each sodium nitrate addition. The three electrodes were left unpolarized for the first four days (102 hours) before being poised at -0.5 V vs. SCE with current recording for the rest of the experiment. The initial concentration of nitrate was set at 400 mg.L⁻¹. Three more

sodium nitrate additions (500 mg.L⁻¹ of NO₃ each) were made in each pilot during the course of the experiment (namely at 190 h, 336 h and 600 h).

2.5. *DNA extraction, 16S rRNA genes amplification and bacterial community study*

After 26 days of incubation, 0.25 g of graphite granules were sampled in the 6 pilots from each compartment of the electrodes (9 samples in total) along with 0.25 g of sediments from the sediment/water interface taken near the electrodes (1 cm). DNA extraction was performed on these 15 samples. The DNA extraction was carried out using the DNeasy® PowerSoil® Kit from Qiagen® following the instructions of the manufacturer. After DNA extraction, the concentration in pure DNA for each of the 15 samples was determined using a quantifying fluorescent dye assay (Qubit dsDNA HS assay kit) software and protocol. Archaeal and bacterial hyper variable region V4-V5 of the 16S rRNA gene was amplified and then sequenced according to a protocol described previously [19,20] with some modifications. V4-V5 of the 16S rRNA gene was amplified by PCR with fusion primers 515F (5'- Ion A adapter-Barcode-GTGYCAGCMGCCGCGGTA-3') [21] and 928R (5'-Ion trP1 adapter-CCCCGYCAATTCMTTTRAGT-3') [22], which include a barcode and sequencing adapters according to the recommendations of the "Ion Amplicon Library Preparation (Fusion Method)". PCR mixture contained 1X SuperFi Buffer, 0.2 mM of each dNTP, 0.5 μM of each primer, 1 U Platinum SuperFi DNA Polymerase (Invitrogen), and 10–20 ng template DNA in a 50 μL reaction volume. Amplification was performed as follows: 30 s at 98 °C, 30 cycles of 10 s at 98 °C, 10 s at 50 °C, 30 s at 72 °C, followed by final extension of 5 min at 72 °C. PCR products were purified using Solid Phase Reversible Immobilization (SPRI) magnetic beads (Mag-Bind TotalPure NGS magnetic beads, Omega Bio-Tek) according to the manufacturer's instructions, with a bead/amplicon ratio of 1.2, and were eluted in 45 μL TE Buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA). Purified amplicons were quantified using D1000 ScreenTape and 4200 TapeStation or DNA 1000 Kit and 2100 Bioanalyzer (Agilent Technologies), following the manufacturer's instructions. Then all amplicons were pre-diluted at 500 pM in molecular grade water and equimolarly pooled. The pool is then diluted at 100 pM for sequencing. Briefly, to prepare template-positive Ion Sphere Particles (ISPs) containing clonally amplified DNA by emulsion PCR, the library was diluted to 26 pM and set up on the Ion OneTouch 2 Instrument (Life Technologies) using the Ion PGM Hi-Q View OT2 Kit (Life Technologies) and following the manufacturer's instructions. These templated ISPs were then purified on the Ion OneTouch ES (Life Technologies) according to the manufacturer's instructions.

Sequencing was performed on Ion Torrent Personal Genome Machine using Ion 316 Chip V2 (Life Technologies) and Ion PGM Hi-Q View Sequencing Kit (Life Technologies) according to the manufacturer's instructions. Sequencing data was processed by the Torrent Suite Software. The software filtered out low quality and polyclonal sequence reads, and quality filtered data was exported as FastQ file.

2.6. *16S rRNA genes sequences library treatment*

The obtained 16S rRNA library in FastQ format was then treated using the FROGS pipeline [23] in order to pre-process sequences (remove those not containing the two primers or with too much unattributed nucleotides), cluster, eliminate chimera, filter the low abundance (keeping only those appearing more than 0.005 % in the whole dataset.) and finally, attribute the sequences left using the silva 132 16S database [24]. For each sample, the number of sequences always exceeded 20 000, which was enough to be considered as reliable for the 16S rRNA study. This step gave only 17% of the total sequences that could be affiliated to one specific species. The rest of the sequences were either multi-affiliated to more than one species or could not be affiliated to more than a bacterial genus (most likely unknown species belonging to this genus). For that reason, the 16S rRNA study are shown only to the genus level.

2.7. *Chemical Analyses*

Nitrate concentrations were determined every two days (S3) through ionic chromatography study on an 881 Compact IC pro (Metrohm®). A control solution (50 mg.L⁻¹) was determined 10 times to calculate the margin of error on the concentration that was always below 5 %. The denitrification efficiency is given in mg of N-NO₃⁻ removed per day and per projected m² of sediments in contact with water. The denitrification efficiency is calculated by the difference of initial nitrate concentration of a given period (400 mg.L⁻¹ for the first period or the result of nitrate determination after a given sodium nitrate addition) divided by the time elapsed per period and by the sediment surface in contact with water (0.0044 m²).

2.8. Statistical analysis

The data concerning denitrification efficiency were collected and analyzed with a one-way analysis of variance (ANOVAs) using the software Rstudio (version 3.6.1). The difference was considered significant for a p -value < 0.05 .

3. Results and discussion

3.1. Chronoamperometry

Three out of six pilots had an assembled electrode that was poised at -0.5 V vs. SCE from the fourth day (102 hours) to the end of the experiment (day 26, 623h) and the current evolution over time was recorded (Figure 2). As a reminder, sodium nitrate (500 mg.L^{-1} of NO_3^-) was added to all pilots at 190 h, 330 h and 600 h. All three cells display a steady state reductive current at the working electrode over the whole course of polarization. This reductive current is most likely caused by oxygen reduction that can occur on graphite at the applied potential -0.5 V vs. SCE [25] (Figure 2). Upon the first addition of nitrate at 190 h, a sharp increase in reductive current is observed in all three pilots (Figure 2) and assigned to a denitrifying and electroactive cathodic biofilm developed at the working electrode. Indeed, abiotic nitrate reduction is not possible at this potential in these conditions (pH range 6 to 8) on bare graphite or stainless-steel electrodes. Close examination of Figure 2 shows that the reductive current increase was delayed for pilot 3 for the first addition of nitrate (green curve, Figure 2). While pilots 1 and 2 still responded well to the second and third nitrate addition, the reductive current measured in pilot 3 exhibited a sudden small decrease of the reductive current around 20 h after the second nitrate injection (336 h) and showed little evolution thereafter. This trend is corroborated by a decrease of nitrate reduction rate. This is tentatively assigned to an unfavorable evolution of the biofilm and pilot 3 will be used as biotic negative control thereafter.

Here Fig. 2

3.2. Nitrate removal efficiency

The denitrification efficiency over the entire experiment was studied for pilots 1 and 2 where denitrifying biocathodes were successfully developed. The experiment was divided in three different periods: (i) the four days before polarization of the electrodes (the 102 first hours), (ii) from the first

nitrate addition (day 8, 190 h) to the second nitrate addition (day 13, 336 h) and (iii) from the second addition to the third one (day 22, 600 h).

As can be seen in Figure 3, left, nitrate removal rates before polarization is comparable for the three pilots with electrodes (1.80, 1.59 and 1.76 g N-NO₃⁻ / day / m² for pilots 1, 2 and 3 respectively). The one-way ANOVA test gave a *p-value* of 0.108 meaning there is no significant difference between pilots with electrodes (not polarized yet) and the pilots without electrode for this first period.

After polarization starts (period ii), a far more effective nitrate removal efficiency is measured at 2.00 and 2.04 g N-NO₃⁻ / day / m² for pilots 1 and 2 respectively (those with a sharp electrochemical response to denitrifying biofilms) whereas pilot 3 (that showed a delayed electrochemical response to the first addition of nitrate) performs less well at 1.39 g N-NO₃⁻ / day / m² during period (ii). This latter value is comparable to those found in period (ii) for the control pilots 4, 5 and 6 (1.35, 1.40 and 1.49 g N-NO₃⁻ / day / m² respectively) that remain on average close to their lower initial value in period (i) (Figure 3). The one-way ANOVA test between the three pilots with polarized electrodes (1, 2 and 3) compared to the pilots without any electrodes (4, 5 and 6) gave a *p-value* of 0.14 meaning there is no significant difference in term of denitrification efficiency. Nevertheless, if we remove pilot 3 (that showed a delayed response for this first nitrate injection) and compare only the pilots 1 and 2 to the 4, 5 and 6, we obtain a *p-value* of 0.0015 indicating that the denitrification efficiency is significantly more effective for those two pilots 1 and 2 compared to the negative controls (pilots 4, 5 and 6). This, together with the above chronoamperometric analyses, indicates the presence of a cathodic electroactive denitrifying biofilm on the polarized electrode of pilots 1 and 2.

After the second nitrate addition starting period iii from day 14 to day 22 (or from 336 h to 600 h), the nitrate removal rate is higher in the pilots with polarized electrodes (pilots 1, 2 and 3) compared to the negative controls. Note that during period iii, the denitrification rate is also slightly higher for pilots 1 and 2 with working electrodes responding to nitrate addition compared to pilot 3 not responding electrochemically to nitrate addition. The statistical study (one-way ANOVA) gave a *p-value* < 0.05 either if pilot 3 is included in the data set of pilots with polarized electrodes (*p-value* = 0.004) or if only the two pilots responding to nitrate on chronoamperometry are included in this data set (*p-value* = 0.0008). Importantly, we note that the nitrate removal efficiency is decreasing overtime for all the pilots (with or without electrodes). This is most likely due to organic matter exhaustion in the surface sediments.

Here Fig. 3

3.3. Study of bacterial communities inside the polarized electrodes

16S rRNA gene amplification and bio-informatics treatment shed light on the composition of the bacterial communities inside the compartments of the three polarized electrodes and in the surface sediments in the six pilots as shown in Figure 4.

1. Study of the bacterial communities in the surface sediments

As shown on Figure 4, the bacterial communities present in the surface sediments for the six pilots are quite different, nevertheless some common features are observed. For pilot 1 (with a polarized electrode reacting to nitrate addition on chronoamperometry) we can observe various communities of bacteria including a single one known to be able to perform denitrification, namely *Azoarcus* [26]. On the other hand, we can also notice the presence of 13 % of sequences affiliated to the Phylum *Planctomycetes* including Genus *Planctomyces* (6 %). The Phylum *Planctomycetes* is known and studied for its anammox metabolism (anaerobic ammonium oxidation), oxidizing NH_4^+ with NO_2^- to produce N_2 gas [27]. The presence of a population able to perform anammox could be linked to a more effective denitrification process performed by the electroactive bacterial community on the electrode causing an increase of the NO_2^- concentration in the first pilot.

In pilot 2 (with polarized electrode reacting to nitrate addition on chronoamperometry) and pilot 5 (pilot without any electrodes), we can observe the presence of largely dominant genus: *Azospirillum*. This genus is well known for being able to perform almost every reactions linked to the nitrogen cycle from fixing inorganic nitrogen but also to produce some N_2 by reducing nitrate and nitrite [28]. Its abundance in the sediments suggest important nitrogen metabolic turnover but it remains difficult to determine which precise nitrogen conversion step(s) are involved.

In the sediment of pilot 3 (that showed a delayed electrochemical response to the first addition of nitrate), we obtain a more diverse population with the noticeable presence of three genera known to contain nitrate reducing species : *Azoarcus* [26], *Stenotrophomonas* [29] and *Pseudomonas* [30,31]. Pilot 4 shows, like pilot 2 and pilot 5, an uneven sequence abundance distribution among microbial community members. The community is indeed largely dominated by sequences from the Genus *Bacillus*. Likewise, this Genus is documented for including many species able to perform denitrification, notably in aerobic conditions which is unusual compared to regular denitrification where NO_3^- is used only in the absence of oxygen as electron acceptor [32].

Finally, in pilot 6 (control without electrodes) sequence abundances appear more evenly distributed among microbial community. Only sequences belonging to Genus *Fusibacter* appear at a level higher than the 5 % threshold. Members of the Genus *Fusibacter* are reported to be able to catalyze sulfate and thiosulfate reducing metabolism [33,34].

Here Fig. 4

2. Study of the bacterial communities in the electrodes compartments

• Electrode from Pilot 1

In the higher compartments of the electrode incubated in pilot 1 (Figure 5), three genera have a significant relative abundance: *Fluviicola* (12 %), *Lutibacter* (6 %) and *Aquimonas* (6 %). *Fluviicola* is a genus containing only a few known species (Supplementary Data File.xlsx) and most of them are not able to perform nitrate reduction. This genus has been found many times in microbial electrodes studies (Supplementary Data File.xlsx) but only a few times with a significant relative abundance (> 5 %), especially in oxygen reducing bio-cathode studies [35,36]. In the second genus, *Lutibacter*, most of the documented species are not able to use nitrate as terminal electron acceptor (Supplementary Data File.xlsx). This genus is rarely found in Microbial Fuel Cell studies and only in very low relative abundance [37,38]. The last genus, *Aquimonas*, only contains one documented species: *Aquimonas voraii* [39]. Although it cannot reduce nitrate, we cannot conjecture on the nitrate reduction ability of the *Aquimonas* species found in the cathodic biofilm given the poor knowledge currently available on this genus.

The middle compartment of this electrode also shows three different genera with a relative abundance of 5 % or more: *Azoarcus* (11 %), *Geobacter* (8 %) and *Lutibacter* (6 %). *Azoarcus* is particularly interesting in our study for two main reasons. First of all, most of the documented species of this genus are able to reduce nitrate and nitrite [26] and, most importantly, this genus has been found in many bio-electrochemical studies [40–44]. The second genus is *Geobacter*, one of the most documented and studied genus including the species with the most efficient extra-cellular electron transfer metabolisms notably *Geobacter sulfurreducens* and *Geobacter metallireducens* [45]. Among the species belonging to the *Geobacter* genus, a noticeable information is that some of them are able to perform electro-chemical denitrification by using an electrode as electron donor [46].

The lower compartment of this first electrode only contains one genus with significant relative abundance which is *Pontibacter*. Among all the documented species found for this genus, only a few are able to perform nitrate reduction [47]. In addition, its predominance in bio-electrochemical studies is documented only in a few research papers [48][49]. Nevertheless, the presence of the *Pontibacter* genus on the two electrodes responding to the three nitrate additions (in Pilot 1 and 2) and its absence from the electrode in Pilot 3 raise the question of its potential role in bio-electro-denitrification.

- Electrode from Pilot 2

As shown on Figure 5, the higher compartment of pilot 2 (responding to nitrate addition on chronoamperometry) shows only one dominant genus: *Pontibacter* already commented above and in the Overview paragraph below. The middle compartment, on the other hand, shows four genera with a significant relative abundance: *Azoarcus* (22 %), *Fusibacter* (9 %), *Flavobacteria* (7 %) and *Simplicispira* (6 %). The relative abundance of *Azoarcus* is quite high and we can note that this genus is both present in the middle compartment of the two electrodes reacting to nitrate addition on chronoamperometry but also shows characteristics expected for species able to perform electro-active denitrification (nitrate reduction metabolism and a suspected electro-activity). The *Fusibacter* genus contains only a few documented species and their nitrogen metabolism remains unknown. They are mostly studied for their sulfur and thiosulfate reducing ability (Supplementary Data File.xlsx). This genus has however been found with an important relative abundance in some bio electrochemical studies [50–52]. The *Flavobacteria* genus contains hundreds of different reported species with many able to reduce nitrate and has been documented in a few papers [43,53,54].

The lower compartment shows two genera: *Pedobacter* (7 %) and *Pontibacter* (12 %). The *Pedobacter* genus contains a significant number of documented species (more than 65) but we can notice that a large majority of those species do not reduce nitrate or nitrite (Supplementary Data File.xlsx). These species are not documented in bio-electrochemical studies to our knowledge.

- Electrode from Pilot 3

On Figure 5, we can observe that in the higher compartment of the electrode in pilot 3 (not responding to the second or third nitrate additions on chronoamperometry), three genera have a significant relative abundance: *Fluviicola* (23 %), *Chryseobacterium* (11 %) and *Stenotrophomonas* (8 %). The *Chryseobacterium* genus includes some documented bacterial species, some being able to perform denitrification and others not (Supplementary Data File.xlsx). There are very few references

reporting this genus in bio-electrochemical studies. The third significant genus is *Stenotrophomonas* that also contains multiple species able to perform nitrate reduction (Supplementary Data File.xlsx). Moreover, this genus is quite well known and documented in the MFC research field for the electroactivity of some of its species [55] and more specially for their ability to bio-electrochemically degrade azo dyes [56,57].

The middle compartment contains only three genera with a meaningful relative abundance and all of them have been briefly described above: *Fluviicola* (21 %, seen in the high compartment inside electrode from pilot 1), *Simplicispira* (15 %, seen inside the middle compartment in electrode from pilot 2) and *Chryseobacterium* (8 %, seen inside the high compartment in electrode from pilot 3).

The last compartment, the lower one of the electrode from pilot 3, shows the presence of the *Fluviicola* genus (7 %) and *Anaerocella* (5 %). Only one species in the *Anaerocella* genus is documented: *Anaerocella delicata*. This species has been tested negative for nitrate reduction [58] and we are not aware of any bio-electrochemical study reporting this genus.

Here Fig. 5

3. Overview of the bacterial communities on the three electrodes

Table 1 shows an overview of the different genera that can be found in the compartments of the working electrodes in the three pilots. As a reminder, the electrodes from pilots 1 and 2 did respond positively on chronoamperometry to the last nitrate addition (just before the DNA extraction), meaning that bacteria colonizing those electrodes were able to harness electrons from the polarized electrodes and use it to reduce nitrate (Figure 2) while the electrode from pilot 3 showed no reaction to the last addition. However, looking at Table 1, two shared genera are found in the compartments of the electrodes from pilots 1 and 2. Those two genera are *Pontibacter* in the bottom compartment (and in the higher for electrode in pilot 2) and the genus *Azoarcus* present in the middle compartment. Although most of the documented species belonging to the *Pontibacter* genera are not able to perform nitrate reduction and although this genus hasn't been reported in many bio-electrochemical studies, it remains possible that *Pontibacter* has a key role in the increase of denitrification efficiency in pilot 1 and 2 compared to the negative control. We can also note that the *Pontibacter* genus is absent from the sediment samples, which could indicate a positive selection pressure for developing on the electrodes and therefore the involvement of *Pontibacter* in the denitrifying metabolism of the mixed cathodic biofilms. The second common genus present in the two electrodes responding

electrochemically to nitrate addition (pilots 1 and 2) and not in the last one (pilots 3) is *Azoarcus*. This genus is quite known in the microbial fuel cell research field, being found in many studies suggesting that it could be electro-active or at least involved in the biofilm electroactivity through electro-assistance of other electroactive species. Moreover, a large majority of the documented species in this genus are able to perform nitrate reduction. We note that this genus is present with a quite high relative abundance (11 % on electrode 1 and 22 % on electrode 2 respectively) in the two electrodes responding electrochemically to nitrate and not in the third one. In addition, the clear increase of the denitrification efficiency (+ 44 %) between the first and second nitrate addition for the two electrodes containing *Azoarcus* (electrode 1 and 2) lend support its potential importance in the increased denitrification in the mixed biofilms. Finally, the specific ways that species from the *Azoarcus* and *Pontibacter* genera may be involved in cathodic denitrification in mixed biofilms (through direct electro-active denitrification, electro-assistance and/or synergistic effects [59,60]) remain open for future studies.

Here Table 1

4. Conclusions

Two efficient denitrifying bio-cathodes were successfully developed through polarization at -0.5 V vs. SCE and showed a significant increased denitrification compared to the negative controls with no electrodes (+ 47.47 % after polarization). *Azoarcus* and *Pontibacter* have been identified as key genera potentially involved in the electro-active denitrification by the mixed cathodic biofilm. Further studies are needed to determine in which way these genera are implicated, through direct or indirect electro-active nitrate reduction or through electro-assistance and synergistic effects. On a more applied perspective, our study confirms the possibility to promote the development of efficient biocathodes from freshwater sediment to enhance nitrate removal from surface waters.

5. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. Acknowledgement

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7. Appendix A. Supplementary data

Supplementary material shows photographs of the compartmented electrode and of the pilots, the abiotic control cyclic voltammetry, the nitrate concentration overtime, an overview of all the statistical ANOVA tests and a survey on the properties (denitrification, electroactivity) of the significant genera found in the 16S rRNA gene sequencing.

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Compartment	Compartment localization									Nitrate reduction for documented species	Found bio-electrochemical Studies reporting this genus
	Bottom			Middle			Top				
	Cell 1	Cell 2	Cell 3	Cell 1	Cell 2	Cell 3	Cell 1	Cell 2	Cell 3		
<i>Pontibacter</i>	11%	12%						13%		Few	Few
<i>Geobacter</i>					8%					Few	Many
<i>Lutibacter</i>				6%			6%			Few	None or Few
<i>Azoarcus</i>				11%	22%					Majority	Many
<i>Aquimonas</i>							6%			None	None or Few
<i>Fluviicola</i>			7%			21%	12%		23%	Few	None or Few
<i>Pedobacter</i>		7%								Few	None or Few
<i>Fusibacter</i>					9%					-	None or Few
<i>Flavobacteria</i>					7%					Various	Many
<i>Simplicispira</i>					6%	15%				Majority	None or Few
<i>Anaerocella</i>			5%							None	None or Few
<i>Chryseobacterium</i>						8%			11%	Various	None or Few
<i>Stenotrophomonas</i>									8%	Various	Many

Table 1: Overview of the bacterial genera present in the compartment of the three electrodes of pilots 1, 2 and 3. Each genus with a significant relative abundance (>5 %) has been surveyed in order to determine their ability to reduce nitrate and their presence in microbial electrochemical studies. Details and references of this survey can be found in the Supplementary Data excel file (Survey of denitrifying species.xlsx).

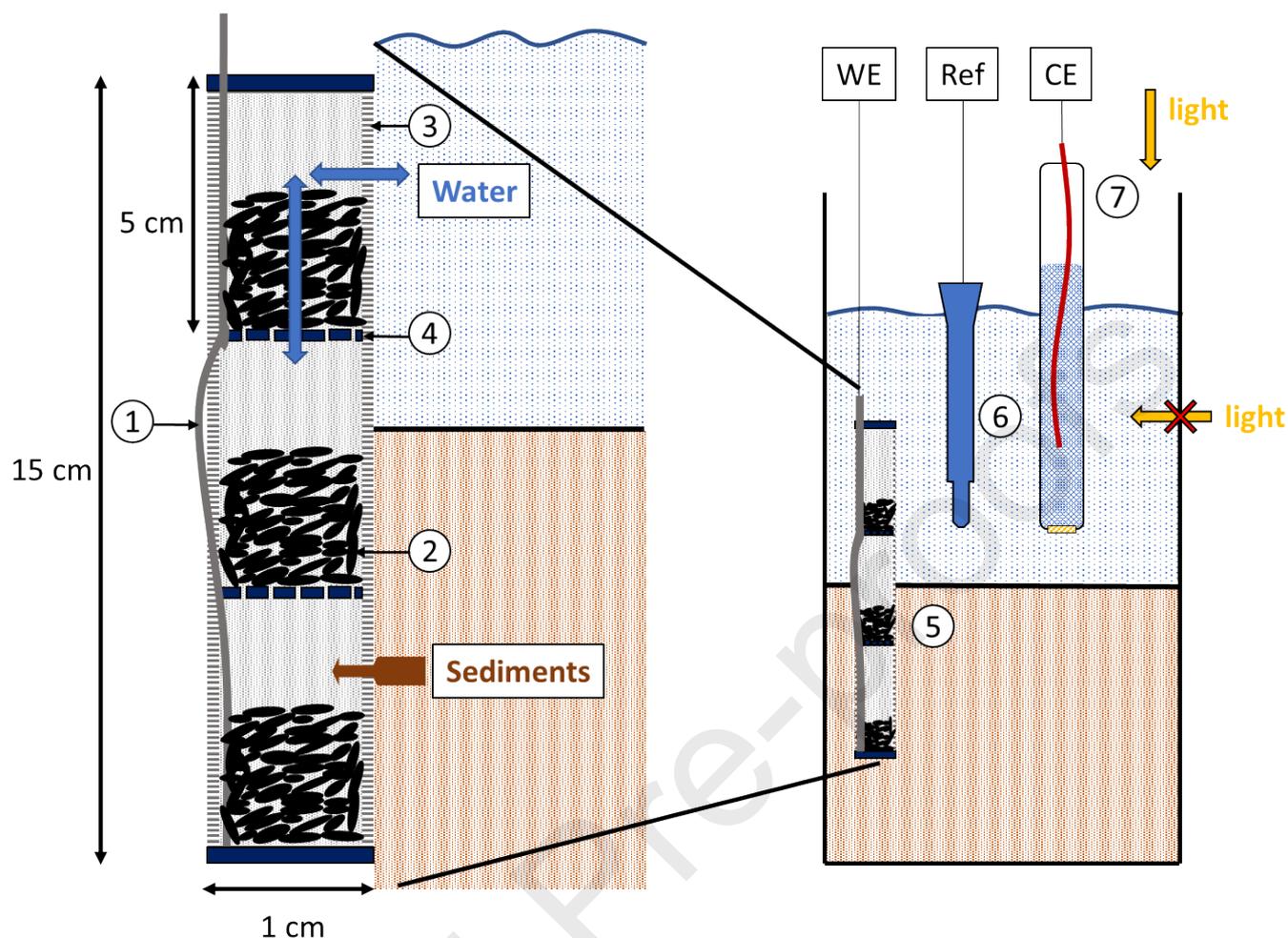


Figure 1. Left: focus on the electrode set-up partially buried in the pilot's sediments: (1) A stainless steel wire for connecting the electrode (2) Graphite granules in each compartment for biofilm development (3) A cylindrical structure made from a stainless-steel grid (4) Plastic separators.

Right: Scheme of the pilot equipped with a three electrodes configuration linked to a potentiostat with (5) the designed electrode as the working electrode (WE) (6) A Saturated Calomel Electrode (SCE) reference electrode (Ref) (7) A platinum wire in phosphate buffer in a separated compartment as the counter electrode (CE). (see also the photographs of the designed electrode and of the pilots in the supporting material)

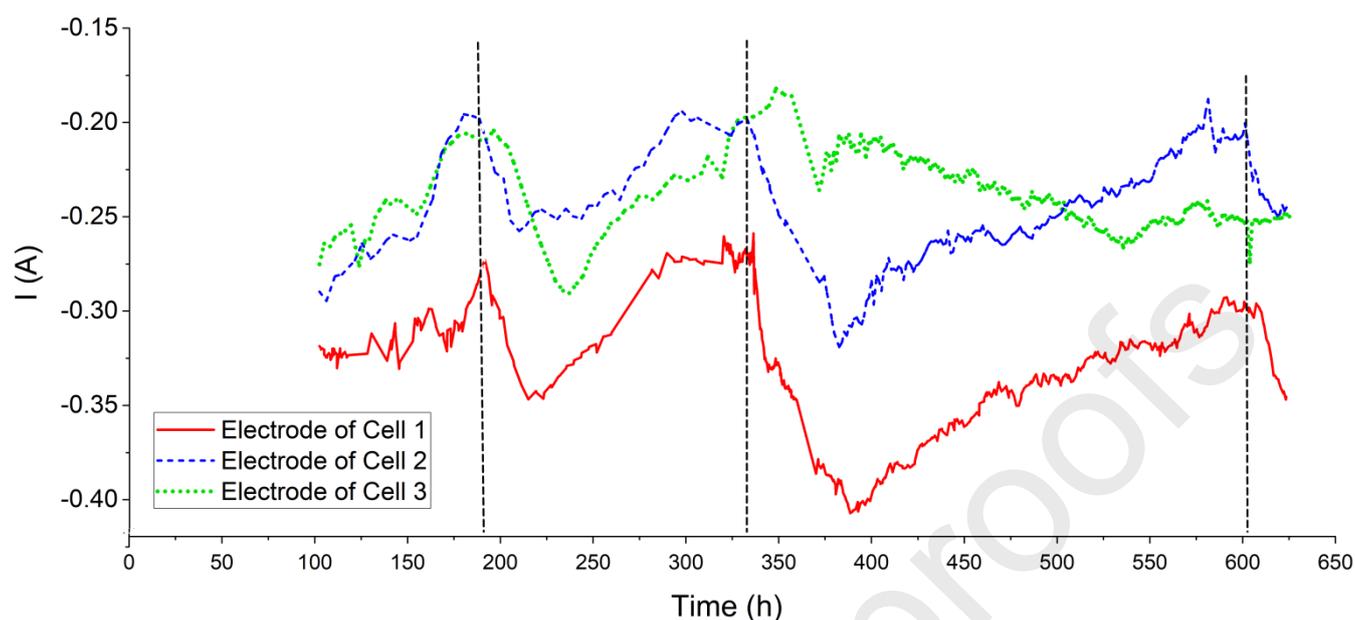


Figure 2. Current vs. time evolution at the electrodes incubated in the pilots 1, 2 and 3. The potential was set at - 0.5 V vs. SCE from day 4 to the end of the experiment at day 26 (623 h). The initial concentration of nitrate was set at 400 mg.L⁻¹. Three more sodium nitrate additions (500 mg.L⁻¹ of NO₃⁻ each) were made in each pilot during the course of the experiment (namely at 190 h, 336 h and 600 h).

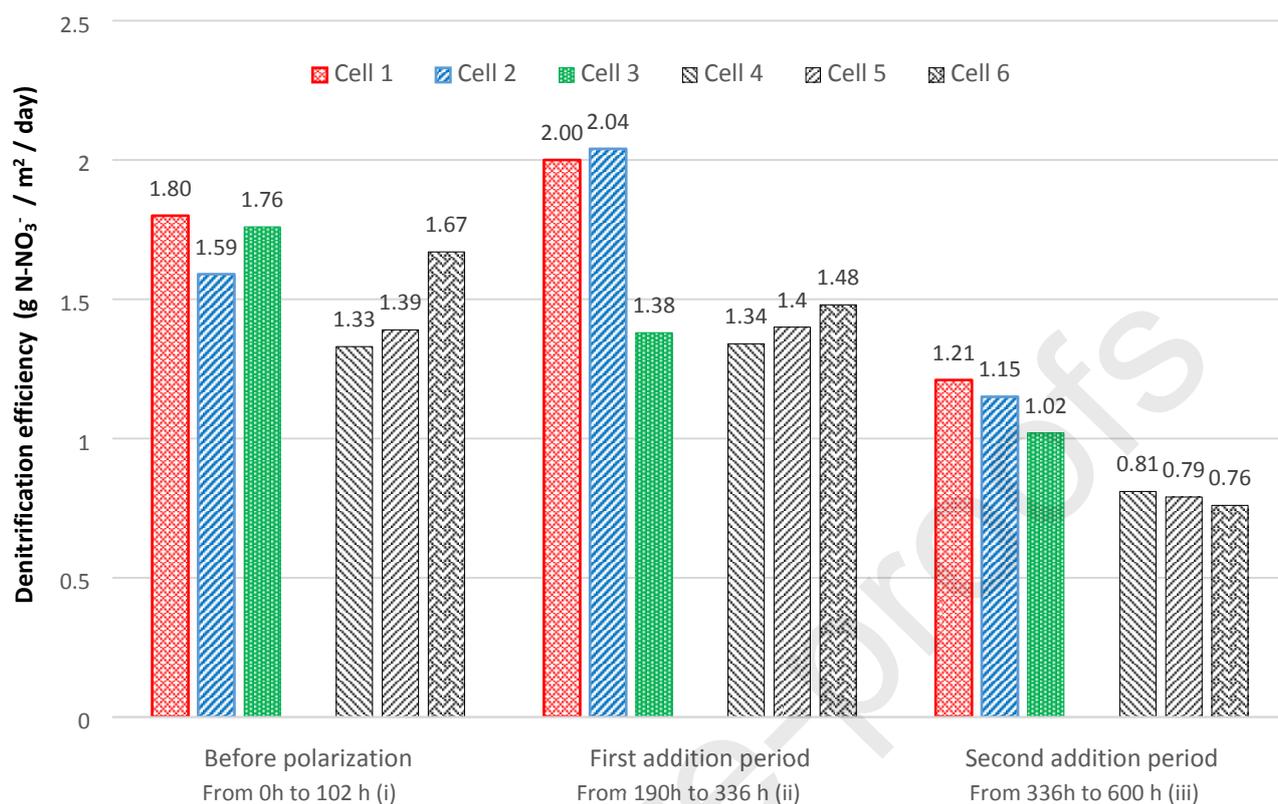
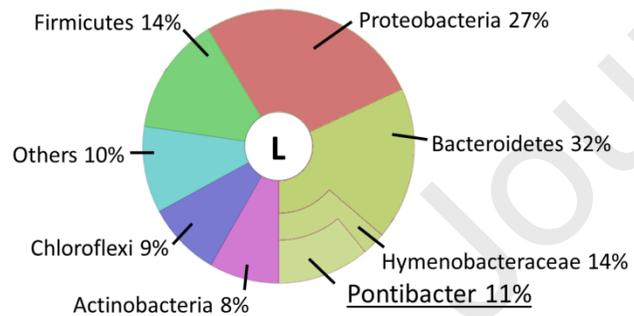
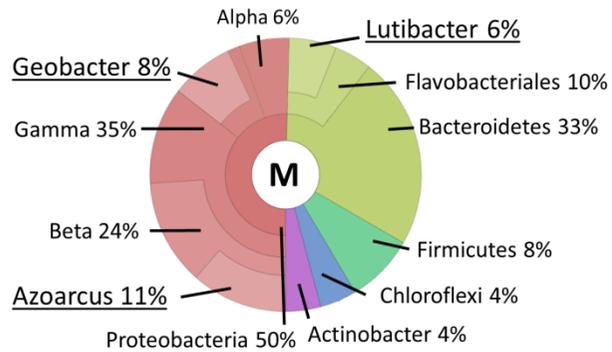
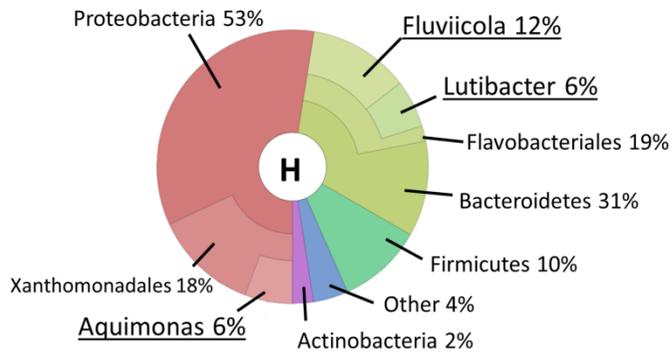


Figure 3: Nitrate removal efficiency on the three periods of the experiment in g of N-NO₃⁻ removed per day and m² of sediments (the surface for each pilot was 44.15 cm²). The total experiment time was divided in three different periods: (i) before polarization, (ii) between first and second sodium nitrate addition and (iii) between second and third sodium nitrate addition.

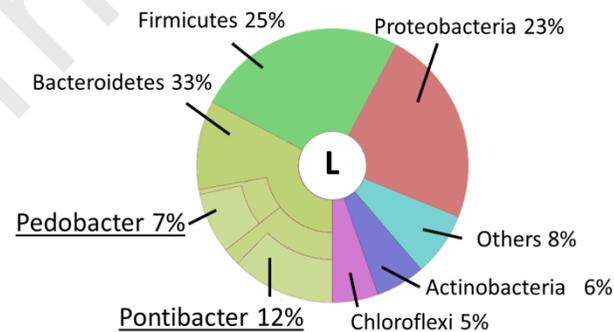
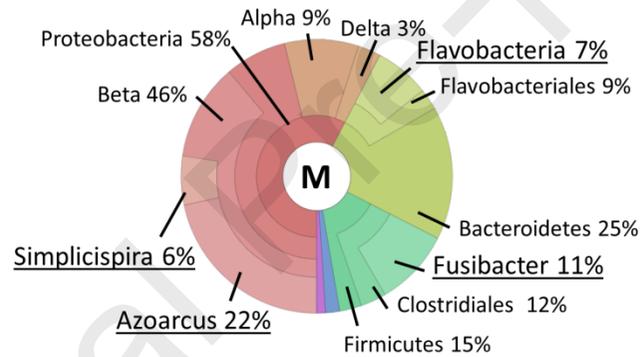
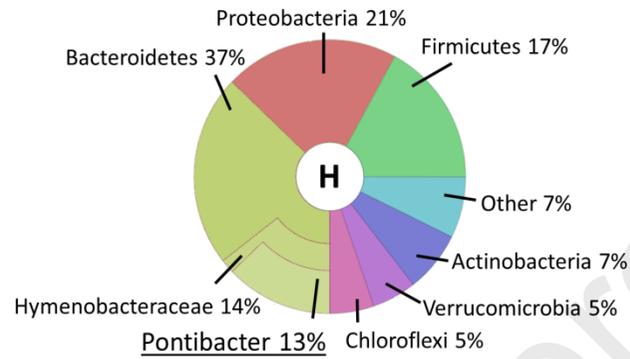


Figure 4: Representation of the bacterial communities' composition from phylum to genus in the surface sediments near the working electrodes of the pilot with electrodes (P1, P2 and P3) and in the surface sediments of the pilots without any electrodes (P4, P5 and P6) at day 26 of incubation (623 h). Only genera with a relative abundance above 5 % are shown on the Krona pies.

Cell 1 (response to third nitrate addition)



Cell 2 (response to third nitrate addition)



Cell 3 (no response to third nitrate addition)

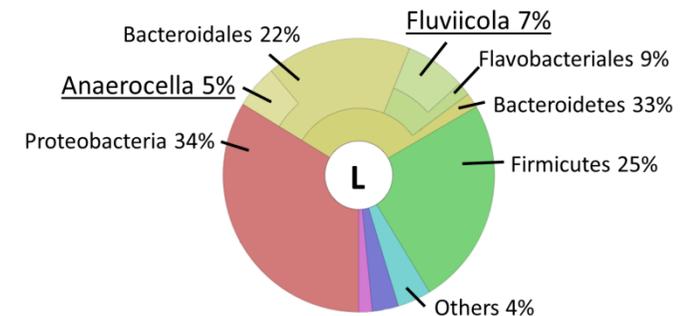
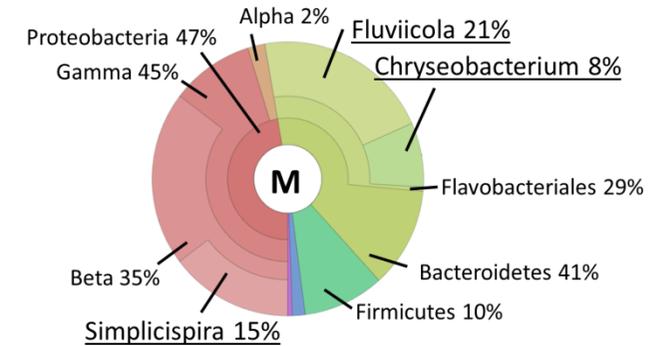
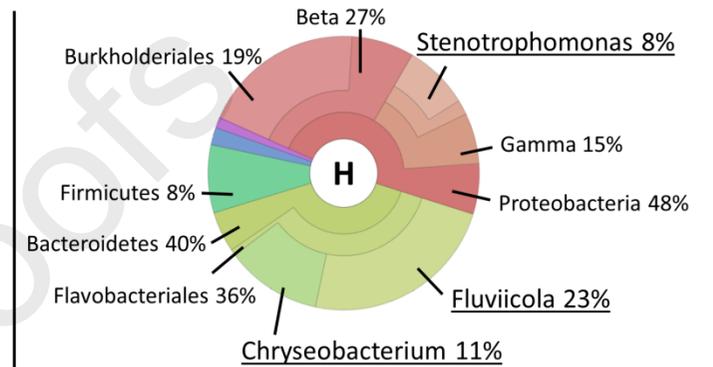


Figure 5: Representation of the bacterial communities' composition from phylum to genus in the three compartments of the working electrode (H for the highest one in water, M for the middle one at the water/sediment interfaces and L for the lower one completely buried in the sediments) from the electrodes of pilots 1, 2 (responding to the three nitrate additions) and 3 (delayed response on chronoamperometry for the first nitrate addition and no significant response to the second and third nitrate addition) at day 26 of incubation (623 h). Only genera with a relative abundance above 5 % are shown on the Krona pies.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights for:

Denitrifying bio-cathodes developed from constructed wetland sediments exhibit electroactive nitrate reducing biofilms dominated by the genera *Azoarcus* and *Pontibacter*

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- Efficient denitrifying biocathodes in sediment from constructed wetland are reported
- Denitrification increased by 45% after 1 month of polarization at -0.5V vs. SCE
- 16S rRNA gene sequencing of the biofilms and sediment are reported
- Genera *Azoarcus* and *Pontibacter* dominate the denitrifying cathodic mixed biofilms