1. Introduction

Ammonium (NH₄⁺) can accumulate in soil and water and can be detrimental to the environment, particularly water systems. Nitrification, the conversion of NH₄⁺ to nitrite (NO₂⁻) and nitrate (NO₃⁻), is the most extensively used method to oxidize NH₄⁺ in engineered systems. However, nitrification is energetically intensive as it requires oxygen inputs, which can account for a substantial amount of energy usage in wastewater treatment systems. A6 oxidizes ammonium anaerobically using ferric iron [Fe(III)] as an electron acceptor and has also been shown to be an electrode (anode) colonizing bacterium. Results presented here demonstrate that A6, in a pure or enrichment culture, can thrive in microbial electrolysis cells (MECs) by oxidizing ammonium, while using the anode as an electron acceptor. Results also show that current production and ammonium removal increase with the concentration of 9,10-anthraquinone-2,6-disulfonic acid (AQDS), a soluble electron shuttling compound, which is especially noticeable for the pure A6 culture. Electron microscopy of the anode’s surface reveals attached cells in the pure culture MEC; however, over the time of operation there is no formation of a biofilm and the majority of cells are in the bulk liquid, explaining the need for AQDS. Maximum coulombic efficiencies of 16.4% and a current density of 4.2 A m⁻² were measured. This is a first step towards the development of a Feammox bacteria-based bioelectrochemical system for anaerobic ammonium oxidation while reducing electrodes instead of Fe(III).

Equation 1: 
\[
\text{NH}_4^+ + 2\text{H}_2\text{O} \rightarrow \text{NO}_2^- + 8\text{H}^+ + 6\text{e}^-
\]

Equation 2: 
\[
3\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O} + 18\text{H}^+ + 6\text{e}^- \rightarrow 6\text{Fe}^{2+} + 10.5\text{H}_2\text{O}
\]

Equation 3: 
\[
\text{NH}_4^+ + 3\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O} + 10\text{H}^+ \rightarrow \text{NO}_2^- + 6\text{Fe}^{2+} + 8.5\text{H}_2\text{O}
\]
reactors to remove NH₄⁺ via the Feammox reaction is not practical because the large amount of iron oxides required results in the accumulation of iron phases over time in the reactor, which requires removal and disposal. To implement the Feammox process for large scale or long-term continuous-flow reactor applications, the requirement of having to add a solid Fe(III) phase needs to be addressed, and an electrode is a suitable option as it can act as the electron acceptor substituting the need for Fe(III) addition.

Electroactive bacteria have been used in bioelectrochemical systems (BES) to extract energy from different types of electron donors and transfer the electrons to the electrodes (anode). The electron transfer process can be direct or aided by electron shuttles and results in the production of low-density electrical currents. Feammox is a microbially mediated process known to be carried out by an Actinobacterium named Acidimicrobiaceae sp. A6 (A6), which is an iron reducer, a feature present in many electroactive bacteria. Experiments to date have shown that A6 grows on solid Fe(III) phases such as ferricydrate, but it does not grow on dissolved Fe(III) such as ferric citrate or ferric chloride. Furthermore, it has been shown that A6 is an electrode colonizing bacterium linked to current production using either a natural redox potential gradient such as in the case of electrodes placed in wetlands where the anode is placed in the more reduced sediment and the cathode is placed in the more oxidized sediment, or in constructed systems when an external potential is applied. Harnessing A6’s ability to use electrodes in BES such as microbial electrolysis cells (MECs) may result in the removal of NH₄⁺, bypassing the need of supplying an electron acceptor such as Fe(III) (eqn (4)).

\[
\text{NH}_4^+ + 2\text{H}_2\text{O} + 6e^- \rightarrow \text{NO}_2^- + 3\text{H}_2 + 2\text{H}^+ \tag{4}
\]

Although other researchers have proposed that NH₄⁺ can be oxidized to N₂ via Feammox, studies with the pure A6 culture and with an A6 enrichment culture using acetylene and N-15 labeled N (ref. 9) have shown that A6 oxidizes NH₄⁺ to NO₂⁻ as shown in eqn (1).

MECs utilize a small external voltage (0.2–0.8 V) for overcoming the thermodynamic barrier of electrolysis. The potential difference between the anode and the cathode is enough to drive the electron transfer from the NH₄⁺ oxidation reaction. The possibility of a reaction can be determined by calculating its standard free energy (ΔG°) using the Nernst equation (ΔG° = -nFEAE°), where n is the number of electrons transferred during the reaction, F is the Faraday constant (96,485 kJ V⁻¹ mol⁻¹), and AE° is the difference in the potentials between two half reactions, measured in volts (V) (ΔE° = E_{anode} - E_{substrate}). E_{substrate} is equal to 0.07 V for NH₄⁺ oxidation to NO₂⁻ (see the ESI† for calculation details). Hence, to make the reaction feasible, E_{anode} needs to be above 0.07 V vs. the standard hydrogen electrode (SHE).

Since we have recently shown that A6 can colonize electrodes in wetland systems and in MECs, and that by doing so current is produced, the objective of this study was to build on these results and gain new insights into the biocurrent-producing mechanisms expressed by Feammox sp. A6 in MECs, and if so, if the rates of NH₄⁺ oxidation and A6 growth in MECs are comparable to those in a batch reactor with Fe(III) as the electron acceptor. For A6 enrichment cultures in MECs, an additional goal was to analyze the microbial communities in MECs with pure A6 and A6 enrichment cultures. Results presented here are a first step towards the development of a Feammox-based system for anaerobic ammonium oxidation in bioelectrochemical reactors in the absence of Fe(III).

Furthermore, it was recently shown that A6 can degrade pollutants such as trichloroethylene (TCE) cometabolically as well as defluorinate per and polyfluoroalkyl substances (PFAS) via reductive dehalogenation. Hence, showing that one can grow A6 in MECs would, in addition to applications of NH₄⁺ removal, lead to novel methods to utilize this strain for biological degradation of a variety or recalcitrant pollutants in bioelectrochemical reactors without the need to handle solid Fe(III) phases.

2. Materials and methods

2.1 Experimental set-up

MECs were constructed and run in parallel as described by Call and Logan (2011), using a stainless steel mesh as the cathode and a graphite plate as the anode since it is chemically stable. The headspace of each MEC was purged with an 80% N₂ and 20% CO₂ gas mixture, and autoclaved. MECs were connected in parallel to a programmable power supply (model 3645A; Circuit Specialists Inc.) with a constant external applied voltage (Vapp) set at 0.3 V. Voltage was recorded hourly with a multimeter (model 2750; Keithley Instruments Inc.) across a 10 Ω resistor placed between the lead connecting the anode and the positive terminal of the power supply. Current (I) was calculated using Ohm’s Law (I = V/R), where V is the voltage and R is the resistance. Data are reported as the volumetric current density (Iq = A m⁻²) which was obtained by dividing the current by the liquid volume in the MEC.

2.2 Cyclic voltammetry

To determine the oxidation peaks for the Feammox reaction in the MECs, cyclic voltammetry (CV) was conducted on the anode, with the cathode as the counter electrode, and a 1 mm thick Ag/AgCl 3.5 M KCl reference electrode (model ET072-1mm, EDaq Inc.) placed between the working and counter electrodes. The applied potential to the MECs was
cycled using an Ivium potentiostat, using the Ivium software. Three consecutive scans were conducted, which ranged from 
−1 V to +1 V at a rate of 1 mV per second. Only the last 2
scans are shown to avoid overcrowding of the figure. CVs
were conducted on MECs with live pure A6 and A6 enrich-
ment cultures with and without 9,10-anthraquinone-2,6-
disulfonic acid (AQDS), which is an electron shuttling com-
 pound, to determine possible effects of AQDS on the system’s
oxidation peaks. CV was also conducted on MECs with a dead
A6 culture with AQDS, and on abiotic control MECs with and
without AQDS to confirm that the peaks found were the re-
 sult of biotic activity from A6.

2.3 MEC operating conditions

Each MEC was inoculated with an A6 pure or enrichment cul-
ture in a Feammox enrichment medium, with a total volume
of 8 ml. The cultures were maintained and provided by Dr.
Shan Huang, following the protocol described in Huang and
Jaffé (2018).7 The medium contained the following: NH4Cl 5
mM, NaHCO3 0.24 mM, KHCO3 0.71 mM, KH2PO4 0.052 mM,
MgSO4·7H2O 0.41 mM, CaCl2 0.54 mM, vitamin supplement
(ATCC® MD-VS) 0.1 μl 1⁻¹, trace element solution as de-
scribed by Sawayama5 and AQDS 0.15 mM. AQDS was in-
cluded after determining that its addition facilitated electron
transfer to the anode from NH4⁺ oxidation, enhancing both
the amount of ammonium oxidized and hence the current
produced (Fig. 3). Additionally, it has been shown that AQDS
is required to grow the pure A6 culture when Fe(m) is the
electron acceptor,7 while for long-term growth of A6 enrich-
ment cultures, AQDS is not needed.9 Vials contained resazurin
(1 mg L⁻¹) as an indicator of anaerobic conditions. The pH
of the medium was initially set to 5–5.5 because the Feammox
process works best under acidic conditions with pH below 6.3.7,9

Replicates of MECs with working Feammox cultures were
run for each experiment (n = 2 for pure cultures and n = 4 for
enrichment cultures). Three types of controls were set up to
confirm that current production and NH4⁺ removal were the
result of biotic activity: 1) MECs with dead bacteria by
autoclaving, 2) abiotic MECs with enrichment medium with-
out microbial inoculum, and 3) MECs with live bacteria in
Feammox medium without NH4⁺. Furthermore, positive controls
with a live Feammox A6 pure culture were incubated in identi-
cal vials without a working anode or cathode, containing Fe[m]
in the form of 2-line ferrihydrite as the electron acceptor19 for
NH4⁺ oxidation and biomass change comparison. All MECs were placed on a mixing plate at 240 rpm; however, using a mixing plate for this purpose resulted in deterioration over time of the connections in the MECs
due to the shaking of the reactors’ connections. Therefore, to
expand the operational time of the MECs and to avoid noisy
readings generated by movement of the whole reactor on the
mixing plate, a later set-up of the MECs with the pure culture
consisted of magnetic stirring bars placed in each reactor po-
sitioned on a stirring plate, such that only the liquid content
in each MEC moved due to the mixing. Since mixing regimes
can affect any reactor performance, no comparison between
experiments with different mixing regimes is done here. Mixing via stirring is only done to expand the operation time of the MEC and to compare its performance against conven-
tional Feammox incubations with Fe[m] as the electron accep-
tor. When current production decreased, the headspace was
flushed with an 80% N₂ and 20% CO₂ gas mixture to ensure
that CO₂ was not a limiting factor as A6 is an autotroph, and
to remove any H₂ formed, as it has been shown that A6 is ca-
pable of oxidizing it.7 The coulombic efficiency (CE = Cᵢ/Cᵢ ×
100%) was determined as the percentage ratio between the
produced coulombs (Cᵢ), calculated by integrating the mea-
sured current over time (Cᵢ = ∫Idt), divided by the theoretical
amount of C produced from the amount of NH4⁺ oxidized to
NO₃⁻ (Cᵢ = ∆NH₄⁺ × nF), where ∆NH₄⁺ is the change in moles
NH₄⁺ measured, n is the amount of electrons produced from
NH₄⁺ oxidation to NO₃⁻ (6 mM electrons per mM NH₄⁺), and
F is the Faraday constant (96 485.33 C mol⁻¹).

Fig. 1 Cyclic voltammetry scans (1 mV per second) for MECs with and without Acidimicrobiaceae sp. A6, and with (+AQDS) and without (−AQDS) AQDS. Optimal Vₜₐₚ = 0.03 ± 0.025 V vs. Ag/AgCl for cultures with AQDS and −0.11 ± 0.035 V vs. Ag/AgCl for MECs without AQDS.
2.4 Chemical analyses

Samples of 1 ml were taken from the MECs at the beginning and end of each experiment. Each sample was filtered using a 0.2 μm pore size syringe filter and used to measure NH₄⁺ and NO₂⁻ concentrations in a Dionex™ ICS3000 Ion Chromatograph, with a CS-16 column, a CS-16 guard column, and a CERS 500 (4 mm) suppressor for cations and with an AS-22 column, an AG-22 guard column, and an ASRS 300 (4 mm) suppressor for anions. Iron was analyzed to quantify the small amount of Fe that was transferred with the culture seed to the MECs (ESI† Table S1). Total Fe was analyzed by adding 100 μl of the MEC or control culture to 4.8 ml of 1 N HCl and 100 μl of 6.25 M NH₂OH–HCl, then Fe was quantified photometrically using the ferrozine method²⁰ as adapted by Komlos and Jaffé.²¹ Ferrous iron [Fe(II)] was quantified by the direct ferrozine method.

2.5 DNA extraction, quantification and microbial community composition

Total genomic DNA was extracted from 5 ml of a working culture of the MECs and from positive live controls at the end of each operational period. DNA was extracted using a FastDNA® spin kit for soil (MP Biomedicals, USA) following the manufacturer’s instructions with an additional first step in which the bacteria were concentrated by centrifuging the liquid medium for 10 min at 14 000 relative centrifugal force (RCF); the pellet was resuspended in 500 μl of the supernatant and used as the initial substrate for extraction. Total DNA was eluted in 100 μl of sterile water and its concentrations were measured using Qubit 2.0® (Invitrogen, USA). All DNA samples were preserved at −20 °C until further analysis.

Quantification of A6 in the pure culture was carried out via qPCR using an Applied Biosystems StepOnePlus real-time PCR system by amplifying a section of the 16S rRNA gen

![Diagram](https://via.placeholder.com/150)

**Fig. 2** Average current density measured in MECs containing live pure A6 and A6 enrichment cultures, with and without AQDS, and dead cultures with and without AQDS (V_app 0.7 V). The points show the mean and the bars the standard error. Notice that the addition of AQDS has a greater effect on the pure A6 cultures than on A6 enrichment cultures, and no significant effect of AQDS on current production is found in dead cultures.

![Diagram](https://via.placeholder.com/150)

**Fig. 3** a) Average current density measured in MECs containing a pure A6 culture and different concentrations of AQDS added as an electron shuttle (V_app 0.7 V). The points show the mean and the bars the standard error (n = 3). b) Average amount of NH₄⁺ removed at each AQDS concentration. Notice that in the absence of AQDS no change in NH₄⁺ concentration was detected, thus the current measured is the result of electrons extracted from NH₄⁺ oxidation and transferred to the anode aided by AQDS.
using primer set 1055F/1392R (1055F, 5′-ATGGCCTGTTCGTCAG
CT-3′; 1392R, 5′-AAGGGCCGTTGTGAC-3′). Each qPCR mix-
ture (20 μl) was composed of 10 μl of SYBR Premix Ex Taq II
2X (TaKaRa, Japan), 0.8 μl of each forward and reverse primer
at 10 μM, and the DNA template. Thermal cycling was initi-
ated with 30 s at 95 °C, followed by 40 cycles, each cycle
consisting of 5 s at 94 °C, 30 s at 55 °C, and 30 s at 70 °C.
Each qPCR assay was run in triplicate for each sample and
included negative controls and a standard curve; the latter
consisted of serial dilutions of known numbers of copies of
dNA.

In order to determine the microbial community composi-
tion, sequencing and phylogenetic analysis was performed by
Novogene (Beijing, China) as follows: from total genomic
DNA, the variable region V4 of the 16S rRNA gene was ampli-
fied using the primer set 515F/806R (515F: 5′-GTCCACGC
MGCCGCGGTAA-3′/806R: 5′-GGACTACHVGGGTWCTCAAT-3′)
with a barcode following the method of Caporaso et al.
(2011).22 All PCR reactions were carried out with a Phusion®
High-Fidelity PCR master mix (New England Biolabs). Quantifi-
cation and qualification of PCR products was carried out by
electrophoresis on 2% agarose gel. The resulting amplicons
were pooled, purified, and quantified. Sequencing libraries
were generated using a TruSeq® DNA PCR-free sample prepa-
ration kit (Illumina, USA) following the manufacturer’s proto-
col and index codes were added. The library quality was
assessed on a Qubit® 2.0 Fluorometer (Thermo Scientific) and
an Agilent Bioanalyzer 2100 system. Finally, sequencing
was performed on an Illumina HiSeq 2500 platform and 250
bp paired-end reads were generated.

Paired-end reads were assembled by using FLASH
V.1.2.7.23 Raw reads were processed according to a QIIME
V1.7.0 quality controlled process and chimeric sequences
were filtered out using the UCHIME algorithm.25 A total of
31,969 sequences were obtained which were clustered
into operational taxonomic units (OTUs) using UPARSE
V7.0.1001.26 Sequences with ≥97% similarity were
assigned to the same OTUs. A total of 995 OTUs were
produced. A representative sequence for each OTU was
screened for taxonomic annotation using the BLAST algo-
rithm against the 2016 NCBI’s 16S ribosomal RNA se-
quences for bacteria and archaea at an e-value of 1e-5.
A6’s 16S rRNA gene sequence (GenBank 2017 accession
number MG589453) was included in NCBI’s database for
annotation at the family and genus level of the top 100
most abundant OTUs.

2.6 Environmental scanning electron microscopy of the
MECs’ anode
The graphite plate working as an anode of MECs
containing live A6, and one from the autoclaved MEC, both
from MECs operated under stirring for over 1
month, were analyzed using an environmental scanning
electron microscope (Quanta 200 FE-ESEM), following the
instrument’s protocol.

3. Results and discussion
3.1 Cyclic voltammetry analysis
Cyclic voltammetry (CV) results (Fig. 1) show oxidation peaks
for all biotic MECs containing AQDS at ~0.03 ± 0.025 V vs.
Ag/AgCl (3.5 M KCl, +205 mV vs. SHE), and for the experi-
ments without AQDS the peak was slightly shifted toward
more negative values, −0.11 ± 0.035 V vs. Ag/AgCl. Applying
these voltages to calculate the ΔG° for the biotic reactions,
using the Nernst equation, results in −60.78 kJ mol⁻¹ with
AQDS and −14.47 kJ mol⁻¹ without AQDS. The effect of AQDS
on the oxidation peaks does not appear to be ample nor sig-
nificantly different for the pure or enrichment culture; none-
theless, initial MEC analysis with pure and A6 enrichment
cultures (V_app of 0.7 V) clearly showed that AQDS has a
greater effect when added to the pure A6 culture than to the
A6 enrichment culture (Fig. 2). These results are consistent
with other studies which have demonstrated that electron
shuttling compounds, such as natural organic matter, can fa-
cilitate electron transfer from the cell to an electron accep-
tor,27,28 or that certain bacterial cells can enable cell-to-cell
electron transfer.29 In the absence of available organic
matter, analogues, such as quinones, aid the electron trans-
port between the cell and the final electron acceptor30 and
facilitate NH₄⁺ oxidation by aiding electron transfer to the an-
ode (Fig. 3). Therefore, in the absence of natural electron
shuttles, AQDS is a good option to facilitate electron transfer,
including in BES, as it has been shown that graphite anodes
incorporated with microbial oxidants, such as AQDS, can in-
crease their I₄ performance,31,32 as seen in our system. Given
the decreased effect of AQDS on current production in the A6
enrichment culture, it is unlikely that AQDS will be needed
in A6 enrichment cultures grown in MECs or other bio-
electrochemical reactors for an extended time, since organic
matter will accumulate due to cell turnover. In the pure cul-
ture MECs, however, a monotonic increase in ammonium re-
moval and current produced with an increasing AQDS con-
centration was observed (Fig. 3), whereas no current was
produced in the presence of AQDS and in the absence of
NH₄⁺ (ESI† Fig. S1).

Other studies on NH₄⁺ removal using BES inoculated with
sludge from wastewater treatment plants reported current ox-
idation peaks of 0.59 V vs. Ag/AgCl [ref. 33] and 0.53 V vs.
Ag/AgCl.34 The conditions used in the mentioned studies, such
as a pH value of 7.7 (Feammox reaction requires acidic pH),
and the associated microbial community, are different from
the ones presented here. Furthermore, Zhan et al. (2014)33 re-
port a microbial community mainly composed of
Stenotrophomonas, Nitrosomonas, Comamonas and Para-
coccus; there is no mention of Acidimicrobiaceae in their sys-
tem. Vilajeliu-Pons et al. (2018)34 attribute nitrification in
their system to Nitrosomonas, and although they found
Actinobacteria, the phyla to which A6 belongs to, the condi-
tions are not optimal for A6, making it inconclusive that the
Feammox process might have contributed to NH₄⁺ removal in
their experiments. Additionally, oxidation peak values similar
to the ones found for the MECs with A6 (−0.08 vs. Ag/AgCl) have been reported for other MEC systems. Nonetheless, that system set-up and microbial community composition was different to the one reported here, as it contained organic carbon and higher pH, to promote growth of organisms such as *Geobacter* spp. The CV on abiotic and dead controls did not show a peak near the oxidation peak for the biotic reactors. The amplitude of the voltammogram on the reverse scans is believed to be due to a pseudocapacitance behavior of the system. The proposed NH$_4^+$ oxidation reaction in the MECs (NH$_4^+$ + 2H$_2$O → NO$_2^−$ + 3H$_2$ + 2H$^+$) supports the increased amplitude of the voltammogram during reverse scanning, because protons can undergo adsorption and desorption prior to H$_2$ formation during scans.

3.2 MECs with pure *Acidimicrobiaceae* sp. A6 culture

Results show that A6 has the ability to be active in MECs, under constant mixing and with $V_{app}$. Different replicas of the MECs run with the pure A6 culture had $I_d$ peaks at different time points (Fig. 4.a). For example, some peaked in the first couple of days, with a maximum $I_d$ value of up to 3.2 A m$^{-2}$, while other live A6 cultures ramped up slower and never peaked; instead, they showed a stable increase of $I_d$ and then leveled out. Differences in the performances of MECs’ replicas have been reported before, being the most noticeable at the start-up period, however, the reason for such behavior needs further analysis. One possible explanation are stochastic processes, such as colonization of the anode’s surface, which could have affected the microbial community function. Although MECs were operated for 3 weeks, $I_d$ data are shown only for a 2-week period, because after that, the connections to the electrodes became loose due to the constant shaking, resulting in noisy data. The MECs with a pure culture had a CE of 4.33%. All control conditions, including MECs with dead A6, abiotic MECs, and MECs with live A6 without NH$_4^+$ showed negligible $I_d$ (ESI† Fig. S1).

All MECs containing the live A6 culture removed on average 0.6 ± 0.25 mM NH$_4^+$, which is similar to the amount removed for cultures grown using Fe(III) as the electron acceptor, over the same time period, i.e. 0.63 ± 0.14 mM NH$_4^+$. The

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Fig. 4  a. Average current density measured in MEC replicas with a pure A6 culture. Live A6-a peaked on day 1 while Live A6-b only ramped up on day 5. Dead A6, as well as other control conditions, not shown here to avoid overcrowding of the figure, shows negligible $I_d$. The arrow represents the time of headspace flushing and injection of an 80% N$_2$ and 20% CO$_2$ gas mixture. b. Average NH$_4^+$ removal after 3 weeks of operation, measured from MECs containing dead A6, MECs without Fe(III) with live A6, batch incubations with live A6 cultures using Fe(III) as the electron acceptor, and from the abiotic control. Bars represent the mean and the marks represent the maximum and minimum amount removed.
control conditions showed no removal of NH$_4^+$, in contrast a slight increase in NH$_4^+$ concentration was detected (+0.11 ± 0.09 mM NH$_4^+$) (Fig. 4b), which could be due to desorption of NH$_4^+$ from the solid phase, such as the ammonium sorbed to cell membrane which has an overall negative charge facilitating the transfer of some NH$_4^+$ from the initial bacterial culture to the MECs. NO$_2^-$ concentrations measurements in MECs were below the detection limit. Low detection of formed NO$_3^-$ with respect to NH$_4^+$ removed via the Feammox process has been previously reported,$^{17,19}$ however, a N mass balance in Feammox enrichment cultures was observed when acetylene gas (C$_2$H$_2$) was added,$^{9}$ which stops the loss of N in the form of N$_2$, by inhibiting the reduction of NO$_2^-$ to N$_2$. Abiotic Fe(n) oxidation by NO$_2^-$ has been observed under similar conditions to the ones used during the Feammox process and in the MECs,$^{10,41}$ which results in the reduction of NO$_2^-$ to different N-gas forms. Hence, a control test incubating NO$_2^-$ with Fe(n) while tracking the NO$_2^-$ concentration over time was performed. Results show that NO$_2^-$ disappeared in the presence of Fe(n) (Fig. S2†). The presence of small amounts of Fe that are transferred to the MECs with the bacterial seed (Table S1†) explains why NO$_3^-$ was not detected, as it would have reacted with the transferred Fe(n).

Quantification of biomass from MECs with the pure live A6 culture revealed that the A6 number can be sustained as well as when grown as a pure culture with Fe(n) as the electron acceptor over 3 weeks of operation (2.90 × 10$^9$ ± 2.8 × 10$^9$ copies of DNA per ml in MECs and 3.35 × 10$^9$ ± 1.97 × 10$^9$ copies of DNA per ml in batch culture with Fe(n)).

Mixing of the medium in the MECs facilitates transport of NH$_4^+$ to cells on the electrode and/or of reduced AQDS to the electrode. However, using a shaker for this purpose resulted in deterioration over time of the connections in the MECs. To expand the operational time of the MECs, magnetic stirring bars were placed in each reactor, which were placed on a stirring plate. This change allowed the operation of MECs with the pure A6 culture for over 1 month, with a continuous increase in $I_d$ over time, with up to 4.2 A m$^{-2}$ (Fig. 5). MECs removed an average of 0.66 ± 0.03 mM NH$_4^+$, comparable to pure A6 batch cultures containing Fe(n) as the electron acceptor which removed 0.64 mM NH$_4^+$. Both MECs and control batch cultures sustained similar amounts of A6 biomass (2.11 × 10$^9$ ± 1.33 × 10$^9$ copies of DNA per ml in MECs and 6.97 × 10$^9$ copies of DNA per ml in batch culture with Fe(n)). This set-up resulted in a CE of 16.4%.

In MECs operated with the pure A6 culture, $I_d$ values of up to 3.2 A m$^{-2}$ were measured in MECs within 2 weeks, and values of up to 4.2 A m$^{-2}$ were measured in MECs that operated for over 1 month. These values are on the lower side of $I_d$ measured in other MEC systems,$^5$ however, their performance cannot be directly compared since their substrates, microbial communities and reactor configurations are different. The highest coulombic efficiency achieved in the MECs was 16.4% which is similar to values found in other studies (CE 9.6–26.4%)$^{43}$ but is on the low side for CE values reported for other systems (CE ≥ 23%);$^{44}$ however, none of those systems are comparable to the ones described here as those other studies contained organic C as their electron donor. The CE values for anaerobic NH$_4^+$ removal in MECs from other studies are 32.7–50%,$^{45,34}$ which are higher than those reported in this study; however their systems also differ from the ones described here in that their microbial communities are composed of a high bacterial diversity, many of which are involved in various steps of the N cycle. A low CE means that the electrons from NH$_4^+$ are not recovered as current, and this could be due to the use of these electrons for the formation of secondary metabolites$^{46}$ or mediators that can be stored and used later,$^{43}$ or to the lack of biofilm formation on the electrode’s surface.$^{47}$ A low CE is also associated with low $V_{app}$, as can be seen from the different $I_d$ values measured in MECs with a $V_{app}$ value of 0.2 V (Fig. 4, 5 and 6) versus MECs with a $V_{app}$ value of 0.7 V (Fig. 2 and 3) which reached higher $I_d$; therefore, applied voltage is a variable that warrants further analysis.

E-SEM revealed bacterial rod-shaped cells, approximately 1.5–3 μm long by 0.5 μm wide, as described for A6,$^7$ attached to the anode’s surface of the MEC with the pure live A6 culture, while no cells could be found on the electrode’s surface of the control MEC with dead bacteria (ESI† Fig. S3†). This result is consistent with a previous study in natural and constructed wetlands showing that the A6 population was enhanced on electrode surfaces working as anodes while no A6 enrichment was found on unconnected plates, thus indicating the need for a voltage difference to promote A6 preference for the anode’s surface.$^{14}$ The formation of a biofilm was not observed during the period and operating conditions of the MECs. Quantification of A6 through qPCR showed that the majority of the cells were in the bulk liquid. This explains why the addition of an electron shuttle like AQDS aided NH$_4^+$ oxidation and current production, as it facilitated the transfer of electrons between the bacterial cell and the electrode surface. The lack of biofilm formation and the presence of AQDS could have also contributed to the low CE, since some AQDS
could have remained in the MECs in its reduced form, without having transferred from the electrode to the anode where it would have produced a current.

3.3 MECs with Acidimicrobiaceae sp. A6 enrichment culture
Since maintaining pure cultures in a biological reactor for extended time periods is a challenge, and since A6 might not remain active in pure cultures for long periods,\textsuperscript{7} we also tested the performance of an A6 enrichment culture in MECs. The goal was to determine if, as for the case of the pure culture, the enrichment culture could also produce current as a result of NH$_4^+$ oxidation. Results for the MECs with the A6 enrichment culture show that MECs produced an average current density ($I_d$) of 2.5 A m$^{-3}$ resulting in a CE of 5.4% and removed a total of 0.52 mM NH$_4^+$ after 3 weeks of operation (Fig. 6).

![Graph showing average current density measured in MECs containing an A6 enrichment culture. The arrow represents the point of headspace flushing and injection of an 80% N$_2$ and 20% CO$_2$ gas mixture.](image)

Fig. 6 Average current density measured in MECs containing an A6 enrichment culture. The arrow represents the point of headspace flushing and injection of an 80% N$_2$ and 20% CO$_2$ gas mixture.

![Microbial community composition from a MEC with an A6 enrichment culture. a) Phylum level. b) Genera in the Actinobacteria phylum, within this phylum, Acidimicrobiaceae sp. A6 has a relative abundance of 47.6%. c) Relative abundance of A6 and other Fe-reducer genera found in the MECs.](image)

Fig. 7 Microbial community composition from a MEC with an A6 enrichment culture. a) Phylum level. b) Genera in the Actinobacteria phylum, within this phylum, Acidimicrobiaceae sp. A6 has a relative abundance of 47.6%. c) Relative abundance of A6 and other Fe-reducer genera found in the MECs.
The microbial community of a working MEC enrichment culture had a relative abundance of 3.83% Actinobacteria, the phylum to which A6 belongs to. This finding is consistent with the relative abundance of Actinobacteria found on electrodes deployed in the field or in constructed wetlands (2–5%). The relative abundance of A6 in these MECs is also similar to that obtained by Huang and Jaffé (2015) for an A6 enrichment culture in a membrane reactor to which ferricydrite was provided as the electron acceptor. Within the Actinobacteria, at the genus level, 47.6% was classified as Feammox bacterium A6, i.e. Acidimicrobiaceae sp. A6, which had a relative abundance of 61.6% of the total diversity of all Fe-reducing bacteria present in the culture (Fig. 7). No other NH$_4^+$ oxidizing bacteria were detected at the genus level (for complete taxonomy of the microbial community composition of the top 100 most abundant genera from a MEC with an A6 enrichment culture see Table S2†).

Actinobacteria is a phylum frequently present in BES but normally is not considered in the microbial analysis of such systems, perhaps due to its lower abundance in comparison with more conspicuous groups such as Proteobacteria, which in the enrichment culture used here had a relative abundance of 78.3% of the total phyla. Proteobacteria is a major phylum that contains a great diversity of bacteria, comprising many widespread ones, including some of the most commonly present and extensively studied electrogenic microorganisms, namely, Geobacter spp. and Shewanella spp. However, regardless of the relative abundance of Proteobacteria in our system, Feammox bacterium A6 abundance made up 61.6% of all Fe-reducing bacteria (FeRB), all of the other genera of iron reducers are Proteobacteria except for Geothrix (Acidobacteria). The other FeRB that are also known to be electrogenic bacteria that can power BES include Geobacter (7.8%), Geothrix (7.2%), and Desulfbolobus (4.7%); however, they require organic C as their electron source and this was not provided to the MECs described here. The only source of C added was CO$_2$ because A6 is an autotroph and we attribute the presence of other FeRB to remnants of the initial microbial community, and any carbon heterotrophic FeBR might be using would come from biomass turnover or any extracellular C produced by A6. Various bacteria, including iron reducers such as Geobacter sp. and A6 (ref. 7), can also use H$_2$ as an electron donor, for this reason the headspace of MECs was flushed periodically, to avoid its interference in NH$_4^+$ removal and overall A6 activity and current production from other bacteria.

There are a limited number of studies on biocatalytic NH$_4^+$ oxidation using electrodes as the electron acceptor, and they conclude that the key organism responsible for this process is Nitrosonomonas (Proteobacteria), a group not found in our MECs. Nitrosonomonas are common aerobic nitrifiers present in soil, freshwater and wastewater. Qu et al. (2014) inoculated their system with freshwater sediments, and Zhan et al. (2014) and Vilajeliu-Pons et al. (2018) inoculated the anode chamber, of a dual-chamber bioelectrochemical system, with sludge from wastewater treatment plants. It is not surprising that they encountered nitrifiers in their systems; however, the mechanism used for anaerobic NH$_4^+$ oxidation by organisms that are normally aerobes, and how they produce current, are still unknown. It has been shown that commonly FeRB, such as Geobacter or Geothrix, have the ability to be electrogenic and thus transfer electrons to an anode, thus producing a current. A6 is an FeRB that carries out Feammox, the only fully anaerobic NH$_4^+$ oxidation process known, hence, we attribute the current production and ammonium oxidation in the MECs in part to A6 activity, especially since no other organism was present in the pure A6 culture MECs. No current was observed in the control MECs with dead A6, abiotic MECs or in the MEC operated with live A6 but without NH$_4^+$.

4. Conclusions

The results presented here demonstrate that the Feammox process, i.e. oxidation of ammonium by Acidimicrobiaceae sp. A6, can be carried out in bioelectrochemical reactors in the absence of ferric iron. A6 biomass can be sustained in equal concentrations in MEC reactors as in batch reactors containing Fe(III), and the relative numbers of A6 in MECs with an A6 enrichment culture were similar to those observed in a Feammox membrane reactor with ferricydrite as the electron acceptor.

Energy production is not the objective of oxidizing NH$_4^+$ in the MECs; the objective is rather to develop an energy efficient method for oxidizing NH$_4^+$ without the need for aeration and as an alternate method to Anammox, and the results presented here show that this is feasible and warrants further development. However, further R&D and process optimization is required for its commercial implementation and application for NH$_4^+$ or co-metabolic trace pollutant removal.

Conflicts of interest

There are no conflicts of interest.

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