Microbial electricity driven anoxic ammonium removal

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Abstract

Removal of nitrogen, mainly in form of ammonium (NH_4^+), in wastewater treatment plants (WWTPs) is a highly energy demanding process, mainly due to aeration. It causes costs of about half a million Euros per year in an average European WWTP. Alternative, more economical technologies for the removal of nitrogen compounds from wastewater are required. This study proves the complete anoxic conversion of ammonium (NH_4^+) to dinitrogen gas (N_2) in continuously operated bioelectrochemical systems at the litre-scale. The removal rate is comparable to conventional WWTP with 35±10 g N m^{-3} d^{-1} with low accumulation of NO_2^-, NO_3^-, N_2O. In contrast to classical aerobic nitrification, the energy consumption is considerable lower (1.16±0.21 kWh kg^{-1} N, being more than 35 times less energy than the conventional wastewater treatment). Biotic and abiotic control experiments confirmed that the anoxic nitrification was an electrochemical biological process mainly performed by *Nitrosomonas* with hydroxylamine as the main substrate (mid-point potential, E_{ox}=+0.67±0.08 V *vs.* SHE). This article proofs the technical feasibility and reduction of costs for ammonium removal in wastewater, investigates the underlying mechanisms and discusses future engineering needs.

Keywords: Bioelectrochemical system, nitrogen cycle, hydroxylamine, microcosm, cyclic voltammetry, wastewater treatment.

1. Introduction

Wastewater treatment is a highly energy demanding process. The removal of organic matter and nitrogen (mainly ammonium; NH_4^+) as the main hazardous products in sewages is necessary for protecting the quality of the water bodies (Duce et al., 2008). Activated sludge treatment is the main process for treating organic matter and nutrients (nitrogen and phosphorus) (van Loosdrecht and Brdjanovic, 2014). During conventional nitrification, ammonium is oxidized aerobically to nitrite (nitritation) and then to nitrate (nitratation) by two functional groups of microorganisms (ammonia-oxidizing bacteria, AOB and nitrite-oxidizing bacteria, NOB). Alternatively, complete nitrification (from ammonium to nitrate) is performed by single microorganisms (van Kessel et al., 2015). Subsequently, nitrate is reduced to dinitrogen gas usually heterotrophically using organic carbon as electron source. The most relevant steps for nitrogen removal are schematically summarized in Fig. A2 (See App, † A3, for details). In wastewater treatment plants (WWTPs) about 4.6 kWh kg^{-1}N are required for aeration (Ekman et al., 2006). This energy consumption sums up to about half a million Euros per year in an average European WWTP of 200.000 person equivalents, with influent flow rates of $5.5x10^4$ m^3 d^{-1} (0.58±0.17 € kg^{-1}N oxidized) (See App, † A2.4, for calculation). This is up to one-third of the total operational cost of a WWTP, without considering the costs of the organic matter addition for denitrification (Horan et al., 1994).

An alternative approach for nitrogen removal is the use of anaerobic ammonium oxidation (anammox) bacteria (Kartal et al., 2004). These bacteria can oxidize ammonium using nitrite as electron acceptor to mainly dinitrogen gas and some nitrate (Fig. A2). The anammox process can

deal with increasing nitrogen loads in a cost effective way in respect to the conventional treatment (Kuenen, 2008; Ni et al., 2012). However, the growth of anammox bacteria is relatively slow: doubling time of 15-30 days, which could be optimized to 3 days when the adequate cultivation conditions are imposed, e.g. low solid retention time (Lotti et al., 2015). Moreover, the limits of the operational conditions are narrow (temperature, pH) and a previous aerobic nitritation process to obtain nitrite is required (Jetten et al., 2009). The anammox technology is established with 109 full-scale installations operating in the world in 2014 (75% for side stream treatment of municipal wastewater)(Lackner et al., 2014), but still the stable supply of appropriate nitrite levels is the most challenging factor (Ma et al., 2016). Moreover, remaining intermediate products as nitrite and nitrate entail additional treatments of the effluent.

Avoiding the disadvantages of the so far established technologies, bioelectrochemical systems (BES) are suggested as an alternative technology also to remove nitrogen (Schröder et al., 2015). In this case, the anode can serve as alternative electron acceptor and substitute oxygen and therewith the cost intensive aeration for nitrification. So far, studies investigating ammonium removal in BES were either performed under aerated conditions (Feng *et al.*, 2015; Zhang *et al.,* 2013; Zhang *et al.*, 2013b; Zhan *et al.*, 2012; Virdis *et al.*, 2010) or were missing controls for oxygen production (Qu *et al.*, 2014) or resulted in an uncomplete nitrogen balance when anoxic conditions were claimed (Zhan *et al.,* 2014). Furthermore, the dependency of the anoxic ammonium removal on electrochemical activity has never been proven and mechanistic knowledge on potential electron transfer reactions and the involved microorganisms is missing so far. In addition to these BES-studies the recent finding of natural anaerobic ammonium

78 oxidation coupled to iron reduction in soils (Feammox, Zhou et al., 2016; Yang *et al.,* 2012)
79 supports the potential of an ammonium removal strategy based on BES.

80 This study shows for the first time that nitrifying BES (niBES) can perform the complete
81 conversion of ammonium to dinitrogen for application relevant ammonium concentrations in
82 continuous mode and under anoxic conditions. Moreover, the electrochemical dependency of
83 the different nitrogen species conversion steps is investigated and the thermodynamics for
84 anodic ammonium oxidation are elucidated.

85

86 **2. Materials and methods**

87 **2.1 Experimental setup**

88 Three replicates of dual-chamber BES reactors (niBES) were constructed identically and
89 operated to remove nitrogen compounds under anoxic conditions (Fig. 1). Each niBES was a
90 two-chamber reactor with granular graphite (model 00514, diameter 4 mm, EnViro-cell,
91 Germany) bed packed anode (electron acceptor electrode) and cathode (electron donor
92 electrode), which decreased the liquid volumes to 0.46±0.01 L net anodic compartment (NAC)
93 and 0.37±0.01 L net cathodic compartment (NCC), respectively (see App, † A2.1, for technical
94 details). Both compartments were separated by an anion exchange membrane (AMI-7001,
95 Membranes International Inc., USA). In order to remove ammonium in the anode, the mixed
96 biofilms were acclimated with anode potential set to +0.8 V *vs.* standard hydrogen electrode

(SHE) applied by a potentiostatic control (SP-50 and VMP3, Bio-logic, France) and pH 7.7, meanwhile the cathode produced hydrogen.

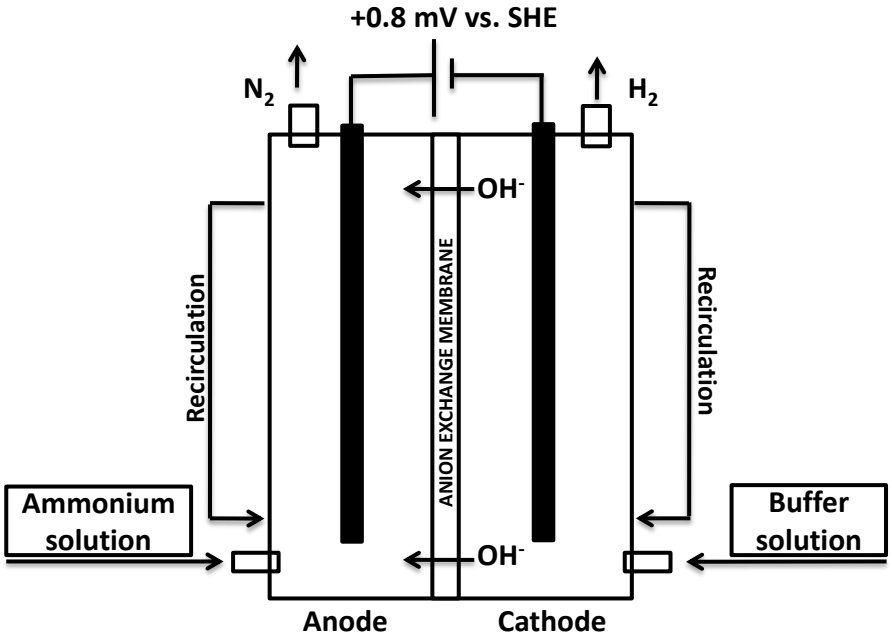


Fig. 1. Schematic diagram of the niBES reactors evaluated in this study.

2.1.1 Inoculation and medium

The anode compartments of the potentiostatically controlled niBES were inoculated with a sludge mixture of an aerobic nitrifying reactor from a wastewater treatment plant (WWTP) in Girona (Spain) (20% of NAC) and a partial nitrifying reactor (20% of NAC) (Gabarró et al., 2012) while the cathodes were inoculated with sludge from a denitrifying BES (40% of NCC) (Pous et al., 2013). In closed-loop mode, a 2 L tank was used during the inoculation. While the anodic compartments contained buffer solution with ammonium and sludge, the cathodic compartments contained only buffer solution with sludge. After 7 days, the sludge from the medium was removed and the operational conditions changed from closed-loop mode to continuous mode (1 L d^{-1}). After 17 days, the current density increased and the ammonium

111 removal in the reactors started, thereby finishing the conditioning phase and marking the
112 beginning of the operational period.

113 The anode and cathode compartment were fed with a buffer solution containing 1.2 g
114 $NaHCO_3$; 10 mM PBS (1.34 g L^{-1} Na_2HPO_4 and 0.30 g L^{-1} KH_2PO_4); 0.5 g NaCl; 0.1 g $MgSO_4 \cdot 7\ H_2O$;
115 0.015 g $CaCl_2 \times 2H_2O$ and 1 mL of trace element solution (Rabaey et al., 2005) per litre of
116 deionized water. The buffer solution of the anode compartment contained additionally 0.39 g L^{-1}
117 NH_4Cl (equivalent nitrogen concentration of 100 mg N L^{-1}). The buffer solution was sparged with
118 helium (He) for 15 minutes prior feeding. An oxygen probe (model 6050, oxygen detection limit
119 0.1 mg O_2 L^{-1}, Mettler Toledo, USA) was introduced in the medium while sparging to ensure
120 anoxic conditions inside the solution (Fig. A1).

121 **2.1.2 Running conditions**

122 After a conditioning phase of 17 days, the reactors were operated potentiostatically under
123 constant flow. Anoxic, helium sparged, buffer solution with 100 mg N L^{-1} of ammonium was
124 continuously supplied at 1 L d^{-1} in the anode compartment. This ammonium concentration
125 corresponds to upper nitrogen peak concentration detected in municipal WWTPs, which
126 normally operate at lower values (below 60 mg N L^{-1}) (Puig et al., 2010).

127 Three different flow rates were applied under constant flow conditions, 1.00, 0.50 and 0.25 L
128 d^{-1} (model 2058, Watson Marlow, United Kingdom) and an internal recirculation loop (60 L d^{-1},
129 model 323 E/D, Watson Marlow, United Kingdom) was placed in each compartment.
130 Accordingly, the nitrogen loading rate was 223±18 g N m^{-3} d^{-1} for the main experimental period
131 (42 days, flow rate 1.00 L d^{-1}), followed by 110±6 g N m^{-3} d^{-1} (27 days, flow rate 0.50 L d^{-1}) and

132 54±2 g N m^{-3} d^{-1} (32 days, flow rate 0.25 L d^{-1}) during the flow rate experiments. The room
133 temperature was kept constant at 23±2 °C.

134 **2.2 Control experiments**

135 Different biotic and abiotic control experiments were performed to confirm the anoxic
136 nitrification as a biological process. The biotic experiments tested the electrochemical
137 dependency of the process and the role of the nitrifying community. For this reason, open-
138 circuit voltage (OCV, without applied anode potential - the anode does not serve as electron
139 acceptor), buffer solution (without electron donor) and specific inhibition (allylthiourea, ATU)
140 experiments were performed.

141 In the OCV tests, the anode potential was changed from closed-circuit voltage conditions
142 (CCV; fixing the anode potential at +0.8V vs. SHE) to OCV for a period of 4 days while all other
143 process parameters were unchanged. As before, the anode compartment was continuously fed
144 at 1 L d^{-1} with buffer solution containing ammonium (100 mg N L^{-1}). The ammonium
145 concentration in the effluent was analysed at the beginning of the OCV period and after 4 days.

146 To investigate the dependency of the current density on the ammonium removal, the
147 influent ammonium concentration was gradually decreased from 100 mg N L^{-1} to 0 mg N L^{-1} by
148 feeding the anode compartment solely with buffer solution without ammonium for 12 days at 1
149 L d^{-1}. The anode potential was kept at +0.8 V *vs.* SHE as before.

150 To test, if the electricity driven anoxic ammonium oxidation was performed by nitrifying
151 microorganisms their activity was inhibited by using allylthiourea (ATU), a specific inhibitor of

the enzyme ammonia monooxygenase (AMO) by chelating copper (Iizumi *et al.,* 1998; Shiemke et al., 2004 and Lehtovirta-Morley *et al.*, 2013). Over 6 days, 0.01 M of ATU (98%, Sigma-Aldrich, USA) was added continuously with the ammonium containing buffer solution to two reactors.

Moreover abiotic experiments were performed in batch mode, applying the same recirculation flow rate as the biotic niBES. Oxygen production and membrane nitrogen transference (NO_3^-, NO_2^-, NH_4^+) were evaluated under OCV and at 0.8V vs. SHE in order to ensure anoxic conditions and measure the potential nitrogen removal from the anode compartment due to diffusion and electromigration (see App, † A4.1 and A4.2, for details).

2.3 Microcosm experiments

For investigating the electrochemical dependency of the different nitrogen species conversion steps tailor-made single-compartment BESs (microcosms) were set up in glass tubes with a final working volume of 15 mL. They contained a working and a counter electrode (graphite rods (CP-Graphite GmbH, Wachtberg Germany) with a projected surface area of 6.68 cm^2) and an Ag/AgCl reference electrode (sat. KCl, SE11 Sensortechnik Meinsberg, Germany). Microcosms were inoculated using 3 mL of inoculum from one niBES anode effluent and 12 mL of fresh medium based on buffer solution and were flushed daily with dinitrogen. The anodic potential was fixed at +0.6 V *vs.* SHE. This anode potential was chosen to be lower than the parent niBES to exclude production of traces of oxygen.

Once a stable current density was reached, the medium was replaced with fresh one to remove planktonic cells and to ensure that the observations were related to the activity of cells attached to the electrode (biofilm). Different buffer solutions were tested containing: i) NH_4^+, ii)

173 NH_2OH, iii) NO_2^-, iv) $NH_4^+ + NH_2OH$, v) $NH_2OH + NO_2^-$, vi) $NH_4^+ + NO_2^-$, vii) $NH_4^+ + NH_2OH + NO_2^-$
174 and viii) buffer solution under anoxic and aerobic conditions. Between the different solutions
175 the setup was rinsed a couple of times with anoxic buffer solution without any nitrogen
176 compounds. All media were applied under anoxic conditions (flushing the microcosm for 10 min
177 with dinitrogen before the experiment was started). Additionally aerobic conditions were tested
178 and an abiotic microcosm (without inoculation) was included as control.

179 Cyclic voltammetry (CV) was applied to identify potential extracellular electron transfer (EET)
180 sites of the microbial cells attached to the anode under all conditions. CV scans were performed
181 at 1 mV s^{-1} in the range of +0.20 V and +0.83 V *vs.* SHE. Four CV cycles were performed in each
182 routine, but only data from the last, steady-state, cycle is shown. CV data was analysed with
183 SOAS software19 to identify the oxidation and reduction peaks (Fourmond et al., 2009).

184 **2.4 Chemical analyses**

185 Liquid-phase standard measurements for nitrogen compounds were performed at regular
186 intervals according to the American Public Health Association guidelines (APHA, 2005). Samples
187 were obtained from the anode and cathode influent and effluent sections of the niBES reactors.
188 The ion concentration was determined using an ion chromatograph (Dionex IC5000, Vertex
189 Technics, Spain) equipped with a conductivity detector. Gas samples were taken from a
190 methacrylate column installed occasionally on the effluent section. The samples were analysed
191 with a gas-chromatograph (7820A GC System, Agilent, Spain) equipped with a thermal
192 conductivity detector (TCD) and Molesieve column (Agilent, Spain) (see App, † A2.3 and A2.4,
193 for details).

2.5 Microbial characterization

For molecular fingerprinting and sequencing samples from one niBES anode compartment were collected from the anode effluent and scratched from the graphite granules surface using a needle. The samples were stored at −20 °C. DNA extraction, PCR amplification, T-RFLP analysis and sequencing of 16S rDNA were done according to Koch et al. (2014). For fluorescence in situ hybridization (FISH), the microbial cells attached to the graphite surface were dislodged in an ultrasonic bath (P-Selecta). After the detachment, the microbial FISH samples were fixed and hybridized (Vilajeliu-Pons et al., 2015). General and specific fluorescent probes were used to characterize the microbial community (see App, Table A6, † A7, for details).

3. Results and discussion

3.1 Ammonium removal performances at different flow rates

All reactors showed anoxic autotrophic ammonium removal of 17±1 mg N L^{-1} which is 35±10 g N m^{-3} d^{-1} (n=3, Fig. 2, Table 1). Gas analyses proved that 97% of removed ammonium in the anode compartment was subsequently transformed to the final product dinitrogen gas (N_2) in the same compartment, over an operational period of 42 days (Table 1). The potential intermediate greenhouse gas nitrous oxide (N_2O) was not detected, while nitrite and nitrate were found only in low concentrations, maximum values obtained of 0.45 mg N L^{-1} and 0.05 mg N L^{-1}, respectively. The increment of the ammonium removal rate (from day 0 to 20, Fig. 2) correlating with the current density (R^2= 0.78, Fig. 3) and, the Coulombic efficiency (*CE*), i.e. the electron

yield (Koch and Harnisch, 2016), of 35±13% demonstrated the microbial electroactivity of the community. Noteworthy, volatilization of ammonium was below 1% of the ammonium removed at the operational pH (7.7). Migration through the anion exchange membrane was non-existent for ammonium and negligible for nitrite and nitrate at the present concentrations (App, † A4.2).

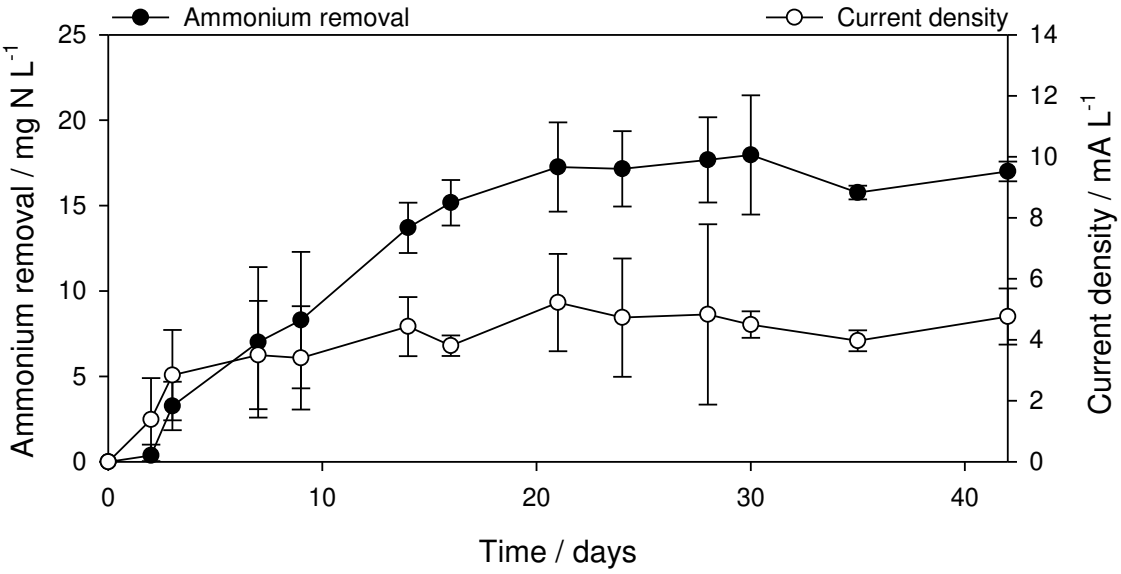


Fig. 2 Evolutions of ammonium removal and current density in the anode compartment of niBES (n=3). The niBES were continuously operated at +0.8 V *vs.* SHE with a constant influent flow of 1 L d^{-1}, during 42 operational days. Values were calculated as the average ± standard deviation of three independent reactors.

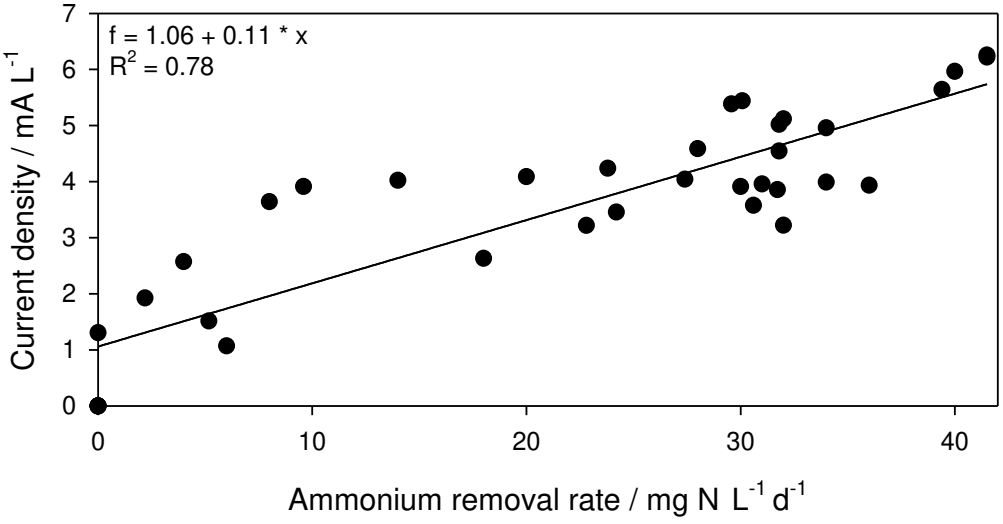


Fig. 3. Correlation between the ammonium removal rate and the current density in the three niBES reactors over 42 days.

Table 1. Nitrogen balances and electric parameters for niBES (n=3) under closed-circuit voltage conditions (CCV; fixing the anode potential at +0.8V *vs.* SHE) and open-circuit voltage conditions (OCV; without applied anode potential). Moreover, buffer solution without ammonium (CCV+buffer) and inhibition of nitrification with allylthiourea (CCV+ATU) were tested. Values are expressed as averages and standard deviations. (n.a.) indicates that compounds were not analysed.

Operational time (days)	Mode	Flow ($L\ d^{-1}$)	Ammonium removal rate ($g\ N\ m^{-3}\ d^{-1}$)	Ammonium removal efficiency (%)	Ammonium removal products (%)				Current			*CE* (%)
					$N\text{-}NO_2^-$	$N\text{-}NO_3^-$	$N\text{-}N_2O$	$N\text{-}N_2$	(mA)	($mA\ m^{-2}$)	($mA\ L^{-1}$)	
42	CCV	1.0±0.2	35±10	17±1	2.6±1.8	0.1±0.1	0±0	97±3	1.9±0.3	3.5±0.5	4.6±0.6	35±13
27	CCV	0.5±0.1	35±6	32±5	1.0±0.5	0.2±0.2	0±0	98±2	1.2±0.5	2.3±0.9	2.7±1.0	28±13
32	CCV	0.25±0.05	9±3	19±5	0.8±0.6	0.3±0.3	0±0	99±1	0.6±0.2	1.2±0.4	1.3±0.4	50±17
4	OCV	1.0±0.1	0.6±0.2	2.5±1.2	0±0	0±0	n.a.	n.a.	-	-	-	-
12	CCV+Buffer	1.0±0.2	0±0	0±0	0±0	0±0	n.a.	n.a.	0.2±0.0	0.4±0.1	0.4±0.1	-
6	CCV+ATU	0.5±0.1	0.5±0.5	0.5±0.1	0±0	0±0	n.a.	n.a.	0.3±0.1	0.5±0.3	0.7±0.2	-

Once all three niBES reached a stable performance (around 42 days), different flow rates (1.00, 0.50 and 0.25 L d^{-1}; Table 1) were compared to better understand the bioelectrochemical ammonium oxidation process for future engineering. Similar ammonium removal rates of around 35 g N m^{-3} d^{-1} were achieved at 1.00 and 0.50 L d^{-1} (Table 1; see App, † A2.4 and A5, for details). However, the highest ammonium removal efficiency of 32±5% was achieved at 0.50 L d^{-1} and the *CE* was the highest at the lowest flow (*CE*s of 50±17% at 0.25 L d^{-1}). The removal rate was reduced to 9±3 g N m^{-3} d^{-1} at 0.25 L d^{-1}. These results showed that, even though the flow increased, the treatment ability was not influenced. Although not directly comparable, the removal rates are twice the number of a previously reported value under batch conditions. In the study of Qu et al. (2014), ammonium conversion to nitrate was found at 17 g N m^{-3} d^{-1} (CE of 33%) working at a similar ammonium concentration. However, the anoxic conditions of the experiment were not ensured. In a different study in which anoxic conditions were ensured 41% of the ammonium was converted to dinitrogen gas at a rate of 12 g N m^{-3} d^{-1} (at 140 mgN L^{-1} influent concentration, CE of 80%, Zhan et al. (2014)) which is only 37.5% of the ammonium removal rate achieved in the current experiments.

However, niBES worked at application relevant ammonium removal rates being in the range of conventional treatments. The ammonium removal was limited to maximum 35±10 g N m^{-3} d^{-1} with the current reactor design due to the appearance of preferential flows inside the compartment (confirmed after computational fluid dynamics (CFDs) modelling) (Vilà-Rovira et al., 2015). The optimization of the hydrodynamic design can improve the ammonium removal rate and treatment efficiency of the niBES. The reduction of dead zones

and preferential flows, together with high surface availability for biomass attachment, will increase the anode performance and are therefore suggested for improved reactor design.

3.2 Reactor performance during different control tests

The idea of a potential bioelectrochemical ammonium oxidation has been stated before (Qu et al., 2014; Zhan et al., 2014) but had been only sparsely investigated to date, e.g. in single reactors without any systematic investigations of the impact of electrochemical oxygen production or the formation of different nitrogen intermediates. Most importantly controls regarding the electrochemical dependency of the process as well as mechanistic knowledge are missing so far. In order to verify the anoxic ammonium oxidation in the anode compartment of the three niBES, respective control experiments were performed. When applying open-circuit voltage conditions (OCV, the anode does not serve as electron acceptor) the observed ammonium removal rate was only 0.6 g N m^{-3} d^{-1}. This value represents 1.7% of the ammonium removal rate under closed-circuit voltage, which was 35±10 g N m^{-3} d^{-1} (Table 1). This shows that there is a clear dependency of the anoxic ammonium removal from the applied anode potential. This provides a clear indication that the ammonium removal was related to microbial electroactivity.

Moreover, when decreasing the influent ammonium concentration gradually from 100 to 0 mg N L^{-1}, the current density decreased also gradually from 4.6±0.6 mA L^{-1} to 0.4±0.1 mA L^{-1} (Fig. 4), supporting that the electrochemical activity was related to ammonium removal (i.e. NH_4^+ serving as electron donor). The background current of 0.4±0.1 mA L^{-1} can result from the buffer solution flowing through the electrode (Bieganowski, 2002) and is not coupled to the ammonium removal. The experiments above confirmed that the electrochemical activity clearly depends on the anoxic ammonium removal.

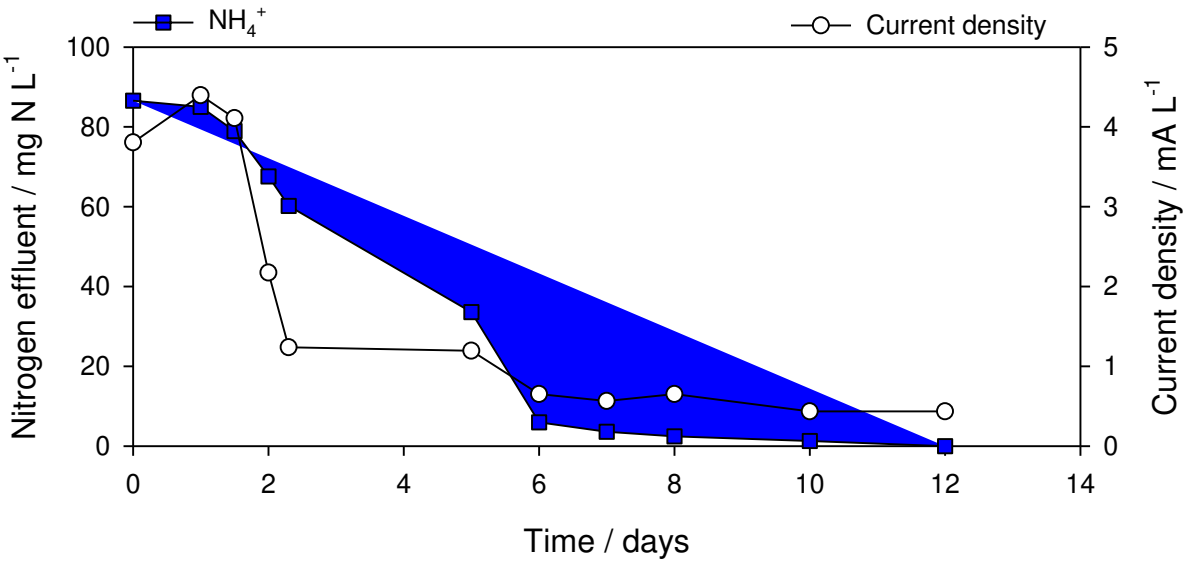


Fig. 4. Ammonium concentration of the effluent and current density after the exchange of media in the influent, from buffer with ammonium (100 mg N L^{-1}) to a buffer without ammonium (0 mg N L^{-1}).

Oxygen was excluded as a potential alternative electron acceptor for the observed ammonium oxidation in the niBES because no oxygen production was detected in the abiotic BES (App, † A4.1).

3.3 Microcosm experiments

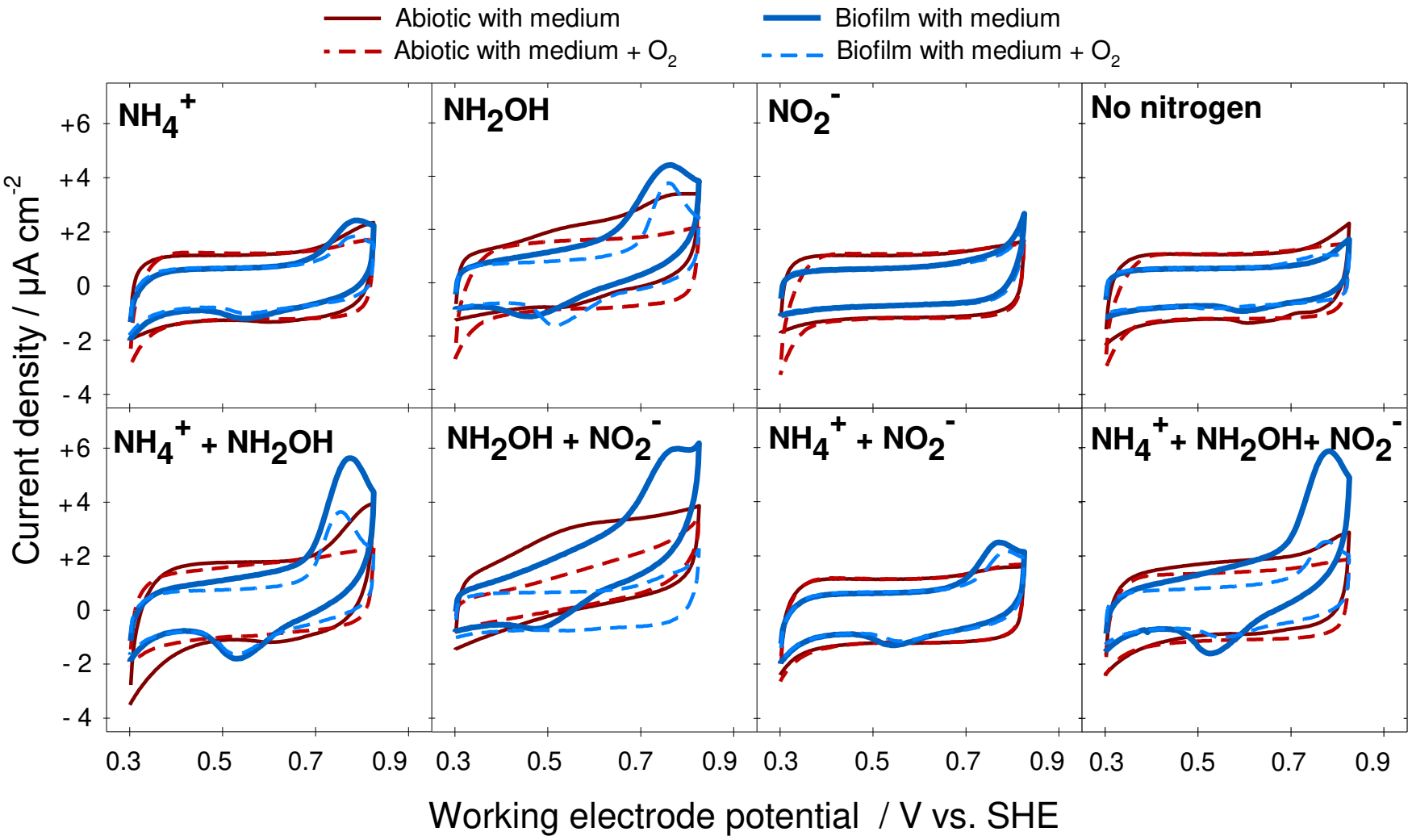
To further investigate the electrochemical dependency of the bioelectrochemical ammonium oxidation as well as a potential electron transfer mechanism, reactor samples of the anode compartment were studied in microcosms, i.e. in tailor-made single-compartment BES Pous *et al.* (2014). After a stable current density was reached, different buffer solutions with a single or several nitrogen compounds (NH_4^+ and/ or its removal intermediates NH_2OH, NO_2^-) or only buffer (without nitrogen species) were tested using cyclic voltammetry under anoxic and aerobic conditions (n=5, App, † A6) including abiotic controls.

The microcosms fed with NH_4^+ under anoxic conditions showed an oxidative peak at +0.73±0.06 V *vs.* SHE and a reduction peak at +0.53±0.03 V *vs.* SHE (Fig. 5, Table A5).

Afterwards, the medium was changed to NH_2OH to investigate the second nitrifying step. In terms of CV analyses, an oxidative peak appeared at +0.67±0.08 V *vs.* SHE and a reduction peak appeared at +0.49±0.06 V *vs.* SHE. The buffer solution with NO_2^- did not result in any peak indicating a lack of electrochemical activity of the NXR enzyme that oxidise NO_2^- to NO_3^-. The buffer solution without nitrogen compounds did not result in any peak (Fig. 5).

The combination of multiple nitrogen compounds as NH_4^+ + NH_2OH in the medium resulted in peaks at the same potentials observed with the respective single nitrogen compounds (NH_4^+ or NH_2OH). The abiotic microcosm confirmed that the oxidation of ammonium and hydroxylamine were not chemically catalysed but biologically.

From these results, it can be concluded that the oxidation of ammonium took place at an oxidative potential of +0.73±0.06 V *vs.* SHE, representing a thermodynamically feasible oxidation cascade to nitrite. It is worth noticing that the maximum current density (4.02±0.46 µA cm^{-2}; n=5) in the microcosms was achieved with NH_2OH as electron donor, whereas NH_4^+ yielded 0.91±0.18 µA cm^{-2} (n=5), and the abiotic control 0.075 µA cm^{-2} (n=1). A potential coupling of ammonium oxidation to an anode was suggested by Zhan et al. (2014) but the study does not provide any information regarding the individual electrogenic conversion steps of ammonium and the intermediate nitrogen species. The results obtained in our study suggest that hydroxylamine, not ammonium, was the main substrate for the electrochemical oxidation performed by the microorganisms attached to the electrodes.



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311 **Fig. 5.** Representative results of the cyclic voltammetries (CVs) performed with a representative biotic microcosm and the abiotic control. Scans were performed at 1 mV s^{-1}
312 while different nitrogen compounds were present (only 4rd scan is shown).

3.4 Microbial community identification

The microbial community of one niBES was characterized based on 16S rDNA analysis.The results show a diverse community that integrated members of different functional groups involved in the nitrogen cycle. Nitrifying bacteria (*Nitrosomonas* genus), Anammox bacteria (*Brocardia* and *Kuenenia* genera), denitrifying bacteria (several *Bacteroidetes* and *Proteobacteria* members), Feammox (*Actinobacteria* members) and *Firmicutes* were identified inside the anode compartment (Fig. 6). The entire diversity of bacteria was certainly not only involved in the ammonium oxidation. We suggest that they contributed to the degradation of intermediate products to dinitrogen (e.g. anammox reaction) but also simultaneous cross reactions as well as transfer of metabolites (e.g. amino acids, organic carbon compounds). This hypothesis is in accordance with the coulombic efficiency (35±13%). The high bacterial diversity is in agreement with previous studies on bioelectrochemical ammonium oxidation, where *Nitrosomonas europaea* was identified as the major contributor to a community together with *Empedobacter* (Qu et al., 2014), or a minor genus in a community dominated by the denitrifying *Thermomonas* (Zhan et al., 2014).

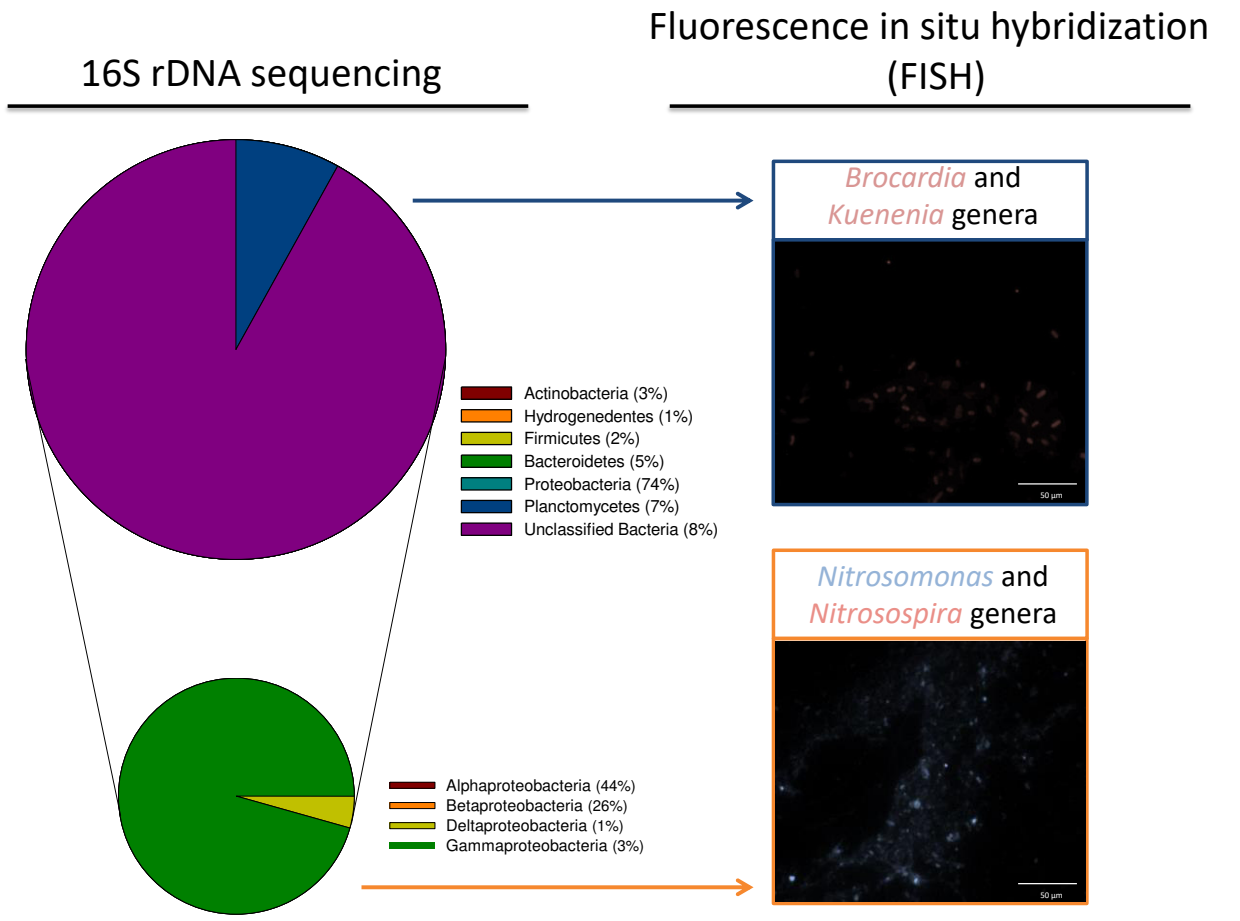

Fig. 6. 16S rDNA analysis of the biofilm in one representative niBES anode compartment. On the left, phyla identified based on sequencing are shown. On the right, representative images of reactor samples after fluorescence in situ hybridisation (FISH) are shown using specific fluorescent probes for anoxic ammonium-oxidizing (anammox) bacteria as *Brocardia* and *Kuenenia* and ammonium oxidising bacteria (AOB) as *Nitrosomonas* and *Nitrosospira*.

To confirm that the anoxic microbial electrochemical ammonium oxidation was performed by nitrifying microorganisms, their activity was inhibited by adding allylthiourea (ATU). ATU is a well-known inhibitor of ammonia-oxidizing bacteria. After adding ATU to the influent, the ammonium removal rate was negligible and the current density decreased from 5.2±0.3 mA L^{-1} to 0.7±0.2 mA L^{-1} (Fig. 7, Table 1). This inhibition lasted for the complete duration of ATU addition (6 days). During this time, also neither intermediate products of ammonium removal

(nitrite or nitrate) nor oxygen or possible oxygen sub-product as oxygen peroxide (H_2O_2) were detected, verifying the microbial electrochemical activity of the nitrifying microorganisms.

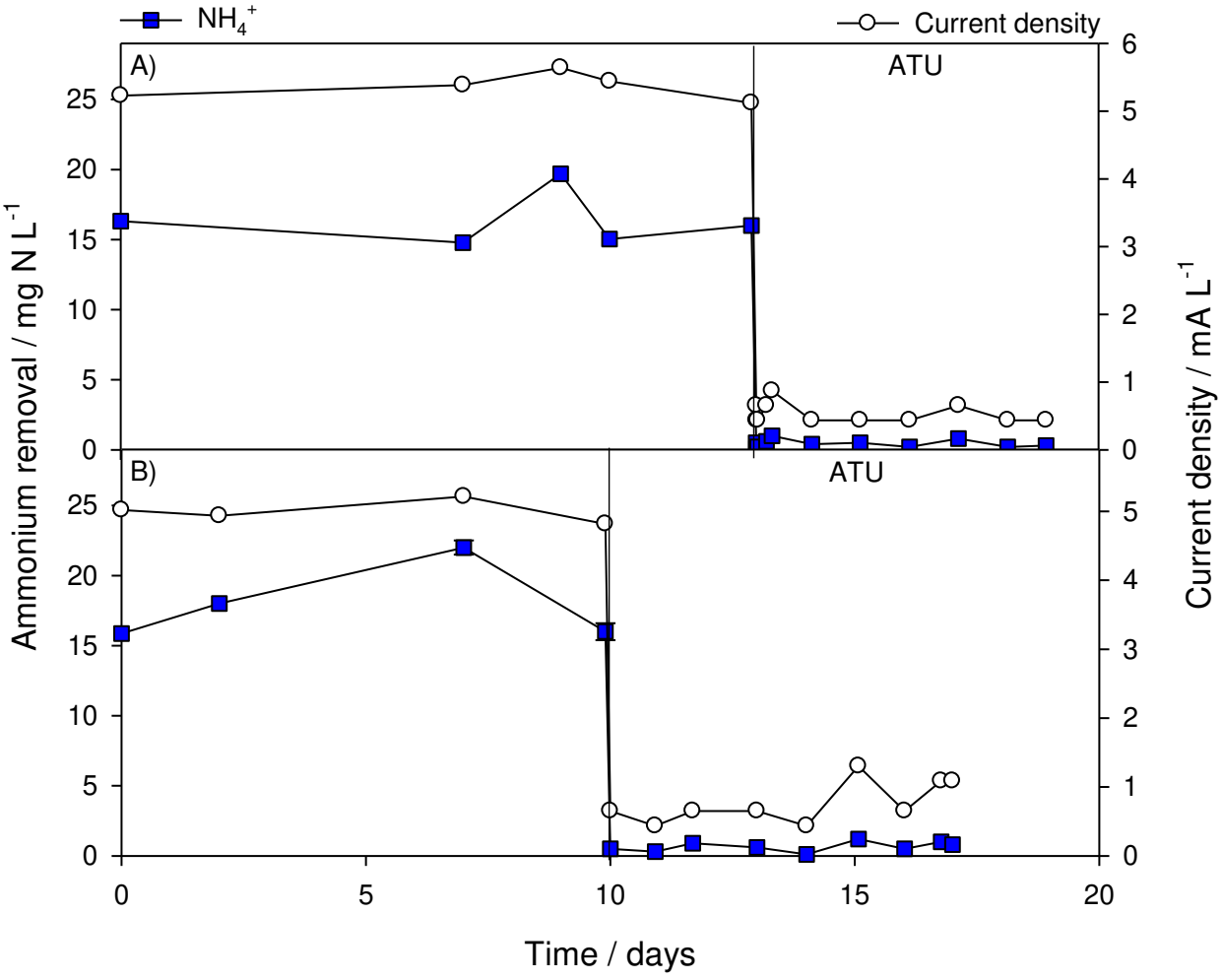

Fig. 7. Evolution of ammonium removal and current density, before and during the addition of the nitrification inhibitor allylthiourea (ATU) in A) niBES 1 and B) niBES 2.

In summary, all experiments clearly showed that electroactive nitrifying microorganisms performed the anoxic autotrophic ammonium oxidation at the anode of niBES with hydroxylamine as the major substrate for the microbially catalyzed electrochemical oxidation. As no accumulation of oxidized nitrogen species was found in any of the reactors further conversions to dinitrogen gas were successfully performed. Due to the microbial diversity in the

350 niBES the contributing reactions were probably a mixture of anammox related reactions (Kartal
351 et al., 2011; van Kessel et al., 2015) and the activity of denitrifying bacteria (by endogenous
352 heterotrophy (Bernat et al., 2008) or by bioelectrochemical denitrification based on interspecies
353 electron transfer or parasitic internal currents, respectively (Harnisch and Schröder, 2009;
354 Rabaey et al., 2010).

355 **3.5 Future applications**

356 From the application perspective, niBES have to be compared to the conventional treatment
357 strategies in terms of costs (Table 2). Both systems, niBES and conventional treatment, present
358 the same removal range and, treat similar amounts of nitrogen equivalents but niBES allowed
359 the transformation of almost all ammonium to dinitrogen gas (>97%) without accumulation of
360 intermediates. The operational costs of niBES were considered for a flow rate of 0.5 L d^{-1} to be
361 0.13 ± 0.09 kWh kg^{-1}N (Table 2; App †, A2.4). Thus, the utilization of a niBES can have a
362 considerable lower energy consumption compared to the classical aerobic nitrification (4.6 kWh
363 kg^{-1}N) (Aymerich et al., 2015). Since low nitrogen intermediate species, as nitrite (0.45 mg N L^{-1}
364 maximum) and nitrate (0.05 mg N L^{-1} maximum), and no nitrous oxide were detected, niBES
365 might further possess a toxicological advantage. One may even speculate that the application of
366 niBES will simplify the reactor operation in WWTPs, as neither air dispersers will not be required
367 for the oxidation of ammonium to intermediates, nor the addition of organic carbon (e.g.
368 methanol) to complete the conventional nitrogen treatment.

369 The niBES are also advantageous compared to the alternative anammox process. 20 years of
370 optimization of the anammox process allowed to reach higher nitrogen removal rates (until 9.52

371 kg N m^{-3} d^{-1}; Phan *et al.,* 2017) as well as reduced energetic costs (1.16 ± 0.21 kWh kg^{-1} N; Wett,
372 2007). However, it requires a previous oxidation of ammonium to nitrite by partial nitritation
373 (Van Dongen et al., 2001), that in turn is based on high hydraulic retention times (between 4-30
374 days) (Wett, 2007) and includes operational challenges (Ma et al., 2016). Therefore, the easy
375 and fast operational mode of niBES makes it a promising alternative technology to current
376 conventional treatments.

377 The further exploration of the anoxic nitrogen removal process in niBES could enhance the
378 treatment capacity of the system in order to be closer to the requirements for application.
379 Different reactor design or increased electrode surface in order to increase biomass attachment
380 and the substrate distribution could improve the nitrogen removal rates (Kim et al., 2014; Vilà-
381 Rovira et al., 2015). Moreover, a better understanding of the underlying molecular mechanisms
382 and balances (e.g. by stable isotope and NanoSIMS analysis) (Musat et al., 2012) in future can
383 help to further improve the process. However, this study already clearly demonstrates a novel
384 and viable methodology to completely convert ammonium to dinitrogen gas under anoxic
385 conditions.

386 **Table 2.** Comparison of energy consumption between bioelectrochemical ammonium oxidation and subsequent complete nitrogen removal under anoxic
387 conditions in this study (flow of 0.5 L d^{-1}) and conventional treatment technologies (aerobic and anoxic). Details on calculations and considerations can be found
388 in App †, A2.4.

Process	N removal rate ($g\ N\ m^{-3}\ d^{-1}$)	Energy consumption ($kWh\ kg^{-1}N$)	Ammonium removal products	Air supply	Addition of products	References
niBES (electricity driven anoxic ammonium oxidation/denitrification)	35 ± 6	0.13 ± 0.09	N_2	No	No	This study
Conventional treatment I (Complete nitrification/denitrification)	21-58	4.6	NO_3^-, N_2	Yes	Sometimes when low C/N ratio, methanol	(Aymerich et al., 2015)
Conventional treatment II (Partial nitrification/denitrification)	21-58	1.6	NO_2^-, N_2	Yes	Sometimes when low C/N ratio, methanol	(Aymerich et al., 2015)
Partial nitritation - Anammox	1200 563 ± 48 9520	—*1 1.16 ± 0.21 —*1	NO_2^-, NO_3^-, N_2 NO_2^-, N_2 NO_2^-, NO_3^-, N_2	Yes Yes Yes	No No Yes, HCl and Na_2SO_3	(Van Dongen et al., 2001) (Wett, 2007) (Phan et al., 2017)

389 *1 : data not available

4. Conclusions

We provide proof-of-concept on a novel technology for the complete anoxic conversion of ammonium to dinitrogen using nitrifying bioelectrochemical systems. The electricity driven anoxic ammonium removal was demonstrated in continuously operated reactors at the litre-scale at a rate of 35 g N m^{-3} d^{-1} and under application relevant flow rates. The electrochemical dependency of the process was confirmed and hydroxylamine identified as the main substrate for the microbially catalyzed electrochemical oxidation. A complex microbial community was detected in the niBES with nitrifying bacteria (*Nitrosomonas*) as key organisms for the anoxic ammonium oxidation. The bioelectrochemical process requires more than 35 times less energy than the conventional process with aeration and provides further advantages since low nitrite and nitrate intermediate nitrogen species were accumulated and no N_2O was detected. Further reactor and process engineering combined with an elucidation of the underlying microbial and electrochemical mechanisms will be needed to even further improvement. Accordingly, niBES are a promising alternative technology for nitrogen removal in wastewater treatment.

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Conflicts of interest

There are no conflicts of interest to declare.

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