Microbial Electrochemical Systems

For Nitrate Removal From Diluted Streams

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Abstract

Increased concentrations of nitrogen compounds comprise one important factor contributing to deterioration of aquatic systems, both engineered and natural environments. In engineered Recirculating Aquaculture Systems (RAS), fish excretion and the addition of excess feed lead to build up of ammonium, which is further converted to less toxic nitrate within a (biological) nitrification unit. Although nitrate can accumulate to concentrations higher than 500 mg L\(^{-1}\) NO\(_3^-\) -N, it is suggested that its concentration should not overcome 50 mg L\(^{-1}\) NO\(_3^-\) -N in freshwater cultures and 100 mg L\(^{-1}\) NO\(_3^-\) -N in seawater cultures. Furthermore, the increased environmental regulations and licencing requirements have further imposed considerably stricter limits for discharge of aquaculture effluents to natural environments (i.e. <3 mg L\(^{-1}\) as Total Nitrogen in Queensland prawn farms). Similarly, other industrial activities and commissioned domestic wastewater treatment plants are also required to treat their wastewater streams to very low (diluted) concentrations of nitrogen before discharging them into natural environments. Therefore, either for recirculation of aquaculture water streams or for discharge purposes of various types of wastewaters, it is recommended that the concentration of nitrogen compounds should be monitored and controlled at low levels.

Since the organic matter present in wastewaters is generally not sufficient to enable complete biological heterotrophic denitrification, typical denitrification processes require addition of an external carbon source (e.g. methanol). Recently, bioelectrochemical cathodic denitrification have been proposed as an alternative to organic matter addition, providing electrons through an inert conductive surface (cathode) directly to a biofilm performing (autotrophic) denitrification. Furthermore, electrons can be generated abiotically by splitting water, which simultaneously oxygenates the water, providing additional benefits for water reuse in aquaculture systems.

Thus, the overall aim of this research project is to develop a Bioelectrochemical system as an alternative technology for denitrification and simultaneous oxygen generation in recirculating streams, which will enable the maintenance of diluted streams with low levels of nitrogen either for recirculation or discharge purposes. Furthermore, the technology should also be applicable as a polishing mechanism for denitrification from other diluted...
streams containing low levels of nitrate, such as secondary (treated) effluents from activated sludge systems.

Since Dissimilatory Nitrate Reduction to Ammonium (DNRA) can be considered an undesired competitive pathway during cathodic denitrification, it is important to demonstrate and quantify the occurrence of this pathway. To assess this phenomenon, a carbon cloth cathodic electrode was inoculated with a mix culture denitrifying microbial community and poised at -0.9 V vs. standard hydrogen electrode (SHE). Results showed that more than 40% of nitrogen added as nitrate was converted via DNRA when the biofilm was at initial stages of development, whereas only 5% was detected as ammonium at later stages of development (7 months of operation), indicating that biofilm age plays a key role on biological pathways occurring during cathodic nitrate reduction. A closer insight revealed that the occurrence of DNRA is linked to cathodic Coulombic efficiency: at low efficiency, a large fraction of the incoming electrons are converted to hydrogen or other reduced compounds within the biofilm, increasing the driving force for DNRA; at high Coulombic efficiency, lower reducing power availability leads to nitrogen gas as preferred reduction product.

Achieving high removal rates is of fundamental importance when designing and scaling up a treatment unit, as it allows for reduction of installation and operation costs as well as the development of a more competitive technology. In order to assess this issue and potentially achieve high denitrification rates, two electrode designs were tested at -0.9 V vs SHE: a 2D graphite plate and a novel 3D Reticulated Vitreous Carbon (RVC - modified with electro-deposited Vulcan carbon particles). The RVC electrodes showed higher nitrate reduction rates and higher current densities than plate reactors when considering the projected surface area of the materials. However, if the total electrode surface area was considered, RVC electrodes then showed a relative lower activity, which indicates that its best performance is in fact due to a bigger surface area available for biofilm growth and not due to the material itself. In addition, the reactors showed an unexpected decrease in activity over operation time, which was attributed to an excessive bacterial growth at such low cathodic potential, which led to a clogging of the electrode and to mass transfer limitations. Those results indicate the need for applying more positive cathodic potentials,
which will reduce cathodic hydrogen formation and consequently reduce the driving force for DNRA, as previously cited, and will avoid the excessive growth/clogging of the biofilm.

A crucial aspect in the applicability of cathodic denitrification at full scale is the reactor setup. In general, bioelectrochemical reactors contain a membrane separating cathodic and anodic compartments, which can considerably increase installation and operational costs. Thus, a single chamber upflow reactor is herein proposed for the treatment of both aquaculture and secondary effluent streams. In this setup, graphite granules are used as both cathode and anode electrodes, allowing for a flow through system. The nitrate-containing stream flows firstly through the cathode electrode placed at the lower portion of the reactor (poised at -0.7 V vs SHE) and only then to the anode electrode in the upper portion of the reactor. Experimental results with synthetic seawater stream operation show that nitrate removal via complete denitrification reached $0.13 \pm 0.01$ kg N m$^{-3}$d$^{-1}$, proving the proposed setup as a viable system for the removal of nitrate from saltwater aquaculture streams. Additional experiments showed that current generation and denitrification rates are strongly dependant on buffer capacity of the water stream.

A similar system was further tested as a polishing mechanism for treated secondary clarified effluent from a municipal wastewater treatment plant, which is characterised by low ionic conductivity ($< 2$ mS cm$^{-1}$) and low concentrations of nitrate ($< 5$ mg L$^{-1}$ NO$_3^-$-N). Nitrogen removal rates reached $0.121$ Kg N m$^{-3}$ d$^{-1}$ depending on the nitrogen loading rate applied, suggesting that biofilm kinetics were not rate limiting.

Finally, an assessment was done on the effects of mixing conditions on ion transfer mechanisms within anode, cathode and gap areas of the upflow setup. The results revealed different ion transfer mechanisms depending on the electrolyte conductivity, in which convection significantly enhances ion transfer during the treatment of freshwater streams, whereas migration plays the main role in the treatment of saltwater streams. Moreover, an assessment was done on the electrolyte potential Ohmic losses, enabling an estimation of a maximum possible cathodic depth in the proposed system.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributions by others to the thesis

Dr Ana Sotres, Ms Alexa von Schledorn and Ms Maria Askildsen contributed to preliminary stages of reactors operation (start-up) in the beginning of this PhD program.

Dr Ludovic Jourdin contributed to experiments design (10%) and provided electroactive biofilm grown on graphite plate, included in Chapter 6.

Prof. Seiya Tsujimura contributed with knowledge on the electrode surface modification technique, included in Chapter 6.

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<td>2D</td>
<td>Two Dimensional</td>
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<td>AEM</td>
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<td>Denitrifying Inoculum</td>
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<td>DNRA</td>
<td>Dissimilatory Nitrate Reduction to Ammonium</td>
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<td>DO</td>
<td>Dissolved Oxygen</td>
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<td>ES</td>
<td>Specialised Electroactive inoculum</td>
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<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>TSS</td>
<td>Total Suspended Solids</td>
</tr>
<tr>
<td>TVC</td>
<td>Total Volume Capacity</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acids</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile Organic Compounds</td>
</tr>
</tbody>
</table>
WE  Working Electrode
WW  Wastewater
WWTP  Waste Water Treatment Plant
1 Introduction

The chemical element nitrogen accounts for approximately 12% of bacteria dry weight, being a fundamental constituent in several organic molecules [1]. Nitrogen is also considered a limiting nutrient, due to the low concentrations usually found in natural environments, which generally determines the growth and spatial distribution of organisms in water courses [2, 3]. As per the nitrogen cycle in nature (Figure 1), nitrogen can be found in different forms, such as organic nitrogen (bonded to organic compounds), ammonia, ammonium, nitrite or nitrate (in solution), or as N₂O, NO and N₂ gases.

Figure 1 – Nitrogen cycle. Blue arrows indicate aerobic processes, while orange, brown and black arrows indicate anoxic processes. The black dashed arrows represents Dissimilatory Nitrate Reduction (DNRA) pathway, also an anoxic process. Adapted from: [4, 5].

Although low concentrations of nitrogen are usually typical of stable environments, the exponential worldwide population growth and increasing food demand and human activities have been modifying this condition. It is noteworthy that the worldwide population is expected to grow from 7.3 billion in 2015 to 8.5 billion by 2030 [6], which will potentially cause a considerable increase in the discharges of organic and inorganic compounds usually present in domestic and industrial wastewaters as well as in agricultural fertilisers over time [7, 8].

Natural systems are able to auto-depurate part of these nutrient discharges without impacting the equilibrium of the waterways. However, a highly increased nitrogen load is
likely to cause eutrophication especially in coastal areas [9], leading to excessive growth of planktonic algae (known as algal blooms) and consequently to ecosystem destruction mainly due to the depletion of dissolved oxygen [2, 10].

Although the removal or minimization of nitrogen from diffuse agricultural sources is a complex issue which is not subject of discussion in this thesis, the treatment of domestic sewage and industrial wastewaters are a common practice done to minimise the environmental impact of point nitrogen discharges. First of all, since ammonium is the most common form of nitrogen in untreated wastewaters [11], its oxidation to nitrate through nitrification (concomitantly to COD removal) prior to its discharge is extremely important to avoid oxygen depletion and fish toxicity in natural waterways. In addition, due to increasingly stricter worldwide environmental regulations (aiming to avoid eutrophication due to nitrate), more recently nitrate removal is also been done prior to discharge of water streams into natural aquatic ecosystems. Very frequently, nitrate removal is done via heterotrophic (biologic) nitrate reduction to dinitrogen gas in a process called denitrification. The Australian and New Zealand Guidelines for Fresh and Marine Water Quality [12] points out that levels of NO\textsubscript{x} (NO\textsubscript{3}^- - N + NO\textsubscript{2}^- - N) ranging from 1-30 µg L\textsuperscript{-1} are already considered trigger values to slightly–moderately disturbed aquatic systems in some tropical areas of Australia. Those values are highly dependent on the level of environmental protection of the aquatic ecosystem and average rainfall, which may considerably influence the licensed discharge limits.

Commonly, the limiting reactant for denitrification in Wastewater Treatment Plants (WWTP) is the organic matter present in the wastewater. Thus, the addition of external carbon sources such as methanol is a common practice, in which the transportation, storage and handling of the flammable material may impose additional costs and safety hazards to the operation. Avoiding the addition of organic matter, bioelectrochemical system (BES) have recently been reported as a promising technology for autotrophic denitrification, which also has the advantage of generating smaller amounts of sludge than the conventional heterotrophic processes such as those carried out in full scale WWTPs. However, there is still a compelling need to improve the obtained nitrate removal rates in BES, as well as to better understand the operation of these systems with waters that
present more realistic characteristics than the commonly tested highly buffered synthetic media.

Thus, this thesis focuses on the treatment of diluted streams such as (1) pre-treated (denitrified) domestic wastewater and (2) recirculating aquaculture streams, in which intrinsic buffer capacity should be enough to allow cathodic denitrification of relatively low concentrations on nitrate. In the first case, it is important to emphasise that a complete nitrate removal via denitrification in activated sludge WWTPs is generally not achieved, as it could significantly increase the required recirculation rates (and aeration costs). Thus, a polishing mechanism for the removal of low levels of nitrate (<10 mg L$^{-1}$) left over from the main denitrification step is sometimes required, in order to avoid pollution in sensitive environments. In the second case, increased levels of water reuse in modern recirculating aquaculture systems (RAS) can lead to accumulation of nitrate coming from fish metabolism and unused feed. If a denitrification step is absent, a high nitrate accumulation may lead to detrimental effects on the production and limit the maximum rates of water reuse. Thus, the establishment of a technology able to treat and maintain low levels of nitrate in recirculating aquaculture tanks is also required. Therefore, this PhD thesis aims to contribute to sustainable environmental practices with the use of BES for the removal of nitrate from diluted water streams for both discharge into waterways and potential water reuse in aquaculture, by deepening the current understanding of the underlying processes and proposing a novel (viable) BES setup.
2 Literature Review

2.1 Diluted Streams: (Pre-treated) Effluent from Activated Sludge Plants

Raw domestic wastewaters typically present high concentrations of organic matter and nitrogen (in ammonium form) [11, 13], as presented in Table 1. Treatment technologies commonly comprise an aerobic activated sludge system for organic matter removal (as represented in the simplified Equation 1) and ammonium oxidation to nitrate (called nitrification, for which the sequence of reactions are demonstrated in Equation 2 and Equation 3). This process can be further coupled to an anoxic denitrification step, able to reduce nitrate to dinitrogen gas [14-16], as represented in Figure 2.

Table 1 – Typical ranges of some different constituents present in the raw (untreated) domestic wastewater, as well as in the effluent from conventional activated sludge and in the effluent from WWTP with nutrient removal step. Adapted from [11].

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Untreated domestic wastewater</th>
<th>Effluent conventional activated sludge</th>
<th>Effluent activated sludge with nitrogen and phosphorus removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS (mg L(^{-1}))</td>
<td>120-400</td>
<td>5-25</td>
<td>5-20</td>
</tr>
<tr>
<td>BOD (mg L(^{-1}))</td>
<td>110-350</td>
<td>10-30</td>
<td>5-15</td>
</tr>
<tr>
<td>COD (mg L(^{-1}))</td>
<td>250-800</td>
<td>40-80</td>
<td>20-40</td>
</tr>
<tr>
<td>TOC (mg L(^{-1}))</td>
<td>80-260</td>
<td>20-40</td>
<td>10-20</td>
</tr>
<tr>
<td>Ammonia nitrogen (mg N L(^{-1}))</td>
<td>12-45</td>
<td>1-10</td>
<td>0.7-3.0</td>
</tr>
<tr>
<td>Nitrate nitrogen (mg N L(^{-1}))</td>
<td>traces</td>
<td>10-30</td>
<td>2-10</td>
</tr>
<tr>
<td>Nitrite nitrogen (mg N L(^{-1}))</td>
<td>traces</td>
<td>traces</td>
<td>traces</td>
</tr>
<tr>
<td>Total phosphorus (mg P L(^{-1}))</td>
<td>4-12</td>
<td>4-10</td>
<td>0.5-2.0</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>-</td>
<td>2-15</td>
<td>2-8</td>
</tr>
<tr>
<td>VOC (mg L(^{-1}))</td>
<td>100-400</td>
<td>10-40</td>
<td>10-20</td>
</tr>
</tbody>
</table>

Equation 1 \( \text{C}_6\text{H}_7\text{NO}_2 + \text{O}_2 \rightarrow 5\text{CO}_2 + 2\text{H}_2\text{O} + \text{NH}_3 + \text{energy} \)

Equation 2 \( 2\text{NH}_4^+ + 3\text{O}_2 \rightarrow 2\text{NO}_2^- + 4\text{H}^+ + 2\text{H}_2\text{O} \)

Equation 3 \( 2\text{NO}_2^- + \text{O}_2 \rightarrow 2\text{NO}_3^- \)
Although urban wastewater treatment is a common practice, the discharge of nitrogen still occur to a certain extent, since the process is not able to achieve 100% nitrogen removal [9]. Thus, although some WWTPs are able to considerably reduce the concentration of N in effluents, a discharge of diluted streams still containing low concentrations of nitrogen (2 - 10 mg L\(^{-1}\) N) can still be expected (Table 1). Considering that a WWTP may have capacity to treat approximately 200 ML per day, the referred N concentration represents a total discharge of 1000 Kg N d\(^{-1}\), still potentially causing significant disturbance to receiving waterways, depending on the local levels of mixing and rainfall.

2.1.1 Nitrogen (nitrate) Removal in WWTP: the need for organic matter addition

Inefficiencies in the removal of nitrogen in WWTP are often related to imbalances in the C/N ratio during the denitrification step [14], when the proportion of organic matter (electron/carbon donor) is not sufficient to sustain stoichiometric requirements for biological denitrification. According to the heterotrophic denitrification reaction with the use of methanol as carbon source (Equation 4), a ratio of 1.9 (mg methanol per mg NO\(_3\)-N) is required [16]. In case acetate is considered as electron donor for denitrification, Equation 5 enables to calculate a ratio of 2.67 mg acetic acid per mg NO\(_3\)-N. In all cases, the equivalent COD/N ratio is approximately 2.86. However, in practice, bigger ratios are required, such as the previously reported proportion of 4.5 [17], which could partly be
attributed to biomass growth [18, 19], or the presence of different forms of COD (i.e. non-biodegradable organic compounds) [20].

Equation 4 \[5\text{CH}_3\text{OH} + 6\text{NO}_3^- \rightarrow 5\text{CO}_2 + 3\text{N}_2 + 7\text{H}_2\text{O} + 6\text{OH}^-\]

Equation 5 \[5\text{CH}_3\text{COOH} + 8\text{NO}_3^- \rightarrow 4\text{N}_2 + 10\text{CO}_2 + 6\text{H}_2\text{O} + 8\text{OH}\]

Equation 6 \[2.16\text{NO}_3^- + 7.24\text{H}_2 + 0.8\text{CO}_2 \rightarrow 0.16\text{C}_5\text{H}_7\text{O}_2\text{N} + \text{N}_2 + 5.6\text{H}_2\text{O} + 2.16\text{OH}^-\]

Even if the influent C/N ratios could theoretically be enough to sustain full denitrification in a WWTP, very high aerobic-to-anoxic recirculation ratios are required to achieve high nitrogen removal efficiencies. The nitrate concentration in the effluent decreases with increasing recirculation ratios. However, this comes at the price of increased energy consumption for pumping and increased return of dissolved oxygen to the anoxic tank. At high recirculation ratios, a sizeable portion of the influent organic matter is oxidised in the anoxic tank by dissolved oxygen coming back with the aerobic-to-anoxic recycle stream, before it can be used as electron donor for denitrification. This may lead to carbon insufficiency and the need to dose COD in the form of methanol, ethanol or acetate to guarantee full nitrification.

Overcoming the need for the addition of a carbon source, autotrophic denitrification have also been studied (Equation 6) [18]. Overall, autotrophic denitrification is advantageous due to smaller biomass formation – an important factor in some systems, since it reduces clogging the reactors – and due to the fact that no organic matter needs to be added, which avoids further carbon contamination into watercourses [21, 22]. It has long been reported to occur in the presence of hydrogen as electron donor and CO\(_2\) as a carbon source [23-27]. However, the low solubility of H\(_2\) in water poses a drawback for its application, since it leads to low efficiency in the use of the added hydrogen [22]. Moreover, the presence of hydrogen constitutes a hazard due to its explosive nature. Although denitrification rates are shown to be higher (almost twice as fast) within a mixotrophic culture in heterotrophic conditions with methanol as electron donor than in autotrophic conditions with hydrogen as electron donor [22], as exposed previously, nitrate may still be encountered in secondary effluents from WWTP, and only limited organic compounds and/or non-biodegradable forms of COD are usually present [20], preventing
the complete heterotrophic removal of nitrate. Therefore, there is still a compelling need for the development of solutions that are able to fully remove N from wastewaters before its discharge into waterways.

2.2 Diluted Streams: Recirculating Aquaculture Systems (RAS)

The worldwide cultivation of aquatic freshwater and seawater species is increasing considerably over the past 30 years [28]. In Australia, approximately 40 different aquatic animal species are known to be commercially produced in aquaculture systems, and over $1 billion production value was reported in 2012-2013 [29, 30].

The setup of an aquaculture farm, the species and stock density of cultured organisms, and the rates of water recirculation will together influence the composition of the water within the tanks [31]. As an example in prawn aquaculture, ponds are usually constructed near a water supply, since the water in the tank fundamentally needs to be replaced at around 10-20% per day [32]. However, more recently, some strategies are being developed in order to avoid or reduce this water exchange, which implies an alteration of water quality inside the tanks, and a subsequent reduction in quality of discharged water – whenever it has to be done. In general, in order to improve internal water quality or to avoid discharge of nutrients and comply with environmental regulations, one or more treatment units are included within an aquaculture system, as indicated in Figure 3.

![Figure 3 – Simplified scheme of a recirculation aquaculture system.](image)

With intensification of aquaculture (i.e. higher fish density) and reduction of water exchange rates (which should ideally comprise only the replenishment of evaporated water or that lost within sludge), more complex systems are being designed which may include various treatment units operated in line or in parallel as represented in Figure 4 from Murray et al [33].

7
As indicated in the Figure 4, solid waste (including fine solids) removal, bio-filtration and carbon dioxide removal are some of the basic treatment units required for the proper operation of a RAS. In addition, continuously monitoring and controlling the system and also disinfecting the tanks after harvesting (before starting new batches, avoiding diseases to be spread) as well as aerating/oxygenating the fish tanks are of fundamental importance. A review of the overall characteristics of aquaculture waters can be found in Table 2 and the main water quality aspects relevant to this thesis are discussed next.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Marine shrimp in low salinity inland water (USA)</th>
<th>Intensive recirculating Aquaculture wastewater</th>
<th>Land based aquaculture wastewater</th>
<th>Marine RAS (Hatchery) seabream and seabass culture</th>
<th>Tilapia during 44 day cultivation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO (mg L(^{-1}))</td>
<td>6.7</td>
<td>-</td>
<td>-</td>
<td>5.1 - 6.9</td>
<td>3.8 - 5.9</td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td>27.9</td>
<td>-</td>
<td>-</td>
<td>16.3 - 28.0</td>
<td>17.0 - 28.0</td>
</tr>
<tr>
<td>pH</td>
<td>8.8</td>
<td>-</td>
<td>-</td>
<td>6.5 - 7.3</td>
<td>6.9 - 7.6</td>
</tr>
<tr>
<td>TAN (mg N L(^{-1}))</td>
<td>0.2</td>
<td>6.8 - 25.6</td>
<td>0.4</td>
<td>0.06 - 6.6</td>
<td>0.1 - 4.6</td>
</tr>
<tr>
<td>NO(_3) -N (mg L(^{-1}))</td>
<td>9.8</td>
<td>11.7</td>
<td>0.09 - 0.2</td>
<td>22.3 - 55.4</td>
<td>25.1 - 62.7</td>
</tr>
<tr>
<td>NO(_2) -N (mg L(^{-1}))</td>
<td>0.3</td>
<td>0.06</td>
<td>0.004 - 0.2</td>
<td>0.1 - 3.37</td>
<td>0.08 - 3.7</td>
</tr>
<tr>
<td>PO(_4)(^3) (mg P L(^{-1}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.4 - 5.0</td>
<td>2.7 - 4.8</td>
</tr>
<tr>
<td>COD (mg L(^{-1}))</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>6.0 - 43</td>
<td>6.0 - 35</td>
</tr>
<tr>
<td>BOD (mg L(^{-1}))</td>
<td>6.4</td>
<td>-</td>
<td>-</td>
<td>12.0 - 16</td>
<td>8.0 - 12</td>
</tr>
<tr>
<td>Chloride (g L(^{-1}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16.5 - 17.8</td>
<td>-</td>
</tr>
<tr>
<td>Salinity (g L(^{-1}))</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
<td>29.8 - 32.2</td>
<td>-</td>
</tr>
<tr>
<td>Cond. (mS cm(^{-1}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>33.3 - 51.1</td>
<td>47.5 - 51.3</td>
</tr>
<tr>
<td>Alk. (mg L(^{-1}) CaCO(_3))</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TSS (mg L(^{-1}))</td>
<td>-</td>
<td>5 - 390</td>
<td>0.4 - 119</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CO(_2) (mg L(^{-1}))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 - 9</td>
<td>1 - 4</td>
</tr>
</tbody>
</table>

*Ref. [34] [35] [36] [37]*

*NITBIO – Nitrifying Biofilm
2.2.1 Water quality and the toxicity of nitrogen compounds in aquaculture systems

The accumulation of nitrogen compounds represents an important factor contributing to water deterioration in recirculating aquaculture systems (RAS). The presence of dissolved nitrogen compounds in fish tanks derives mostly from fish excretion and the addition of excess fish feed [31]. As reviewed by Avnimelech [38], fish or shrimps cultivated in ponds assimilate only approximately 25% of the nitrogen in the feed. Instead, 75% of nitrogen compounds are either (1) excreted as ammonium (NH$_4^+$) [39] or (2) maintained as organic N in faeces or unused feed. Studies on nitrogen budget in recirculation systems which include a nitrifying unit indicate that approximately 30% of nitrogen added as feed and excreted as ammonium is actually further converted to nitrate (NO$_3^-$) [40, 41], whereas 1.7% and 0.6% are found as ammonium (NH$_4^+$) and nitrite (NO$_2^-$), respectively [40]. Furthermore, it was previously shown that the lower the level of water exchange in RAS, the higher the accumulation of NO$_2^-$, NO$_3^-$ and NH$_4^+$ in recirculation systems [42].

Ammonium is known to present high toxicity to aquatic species [31, 43], since it is in constant equilibrium with its un-ionised toxic form (NH$_3$). Thus, the pH of the aqueous media dictates whether the reaction is directed towards one or another compound, as per the following equilibrium [44]:

\[
\text{Equation 7} \quad \text{NH}_4^+ \leftrightarrow \text{NH}_3 + \text{H}^+
\]

The exposure of aquatic species to concentrations of 2, 3 or 4 mg L$^{-1}$ NH$_3$ can lead to high mortality rates (e.g. between 93 and 100% in tilapia) [45]. In fact, the suggested maximum concentration of NH$_3$ in the culture of aquatic species can be as low as 0.05 - 0.27 mg N L$^{-1}$, as reviewed by Eshchar et al [44]. In order to prevent mortality of cultured species in the presence of ammonium, an attempt can be made in keeping a lower pH in the recirculation aquaculture system. In this case, if considering the maintenance of NH$_3$ concentration at around 0.07 mg N L$^{-1}$, a Total Ammonium Nitrogen (TAN) of 1.22 mg N L$^{-1}$ can be maintained at pH 8.2. However, at pH 7 the reaction is shifted towards NH$_4^+$ species, and therefore a higher concentration of TAN (18.26 mg N L$^{-1}$) can be tolerated without increasing the concentration of the toxic form NH$_3$ [44]. However, since this pH control was done with CO$_2$, the authors emphasise that a sudden change in pH cannot be excluded.
and it can lead to a drastic increase in NH₃ concentration and death of cultured species [44]. Because of that, nitrifying treatment units such as a sand filter are usually installed in RAS [46], transforming ammonium into the less toxic nitrate, which may accumulate to concentrations as high as 500 mg L⁻¹ NO₃⁻-N when applying low water exchange rates and increased recirculation rates [47]. Moreover, the accumulation of intermediate nitrite may also occur during nitrification, which can potentially also lead to additional acute toxic effects to fish [43].

Although nitrate is generally tolerated by fish even at considerably higher concentrations than ammonium and nitrite, its presence at concentrations higher than 125 mg L⁻¹ NO₃⁻-N may be harmful to the growth of some species such as turbot and prawns, especially during the initial stages of growth [48, 49]. In addition, considering that in certain conditions of low oxygen and high pH the nitrate could be reduced to ammonia, the Australian and New Zealand Guidelines for Fresh and Marine Water Quality [12] suggest that nitrate concentrations in aquaculture systems should be lower than 50 mg L⁻¹ for freshwater species and 100 mg L⁻¹ for saltwater species. Thus, maintaining the concentrations of nitrogen compounds below those values in RAS is extremely important for a larger and healthier fish production in systems with high water recirculation rates. Furthermore, due to increasingly stricter environmental regulations worldwide, nitrate removal is also required prior to discharge of water streams into natural aquatic ecosystems.

**Nitrate Removal in RAS**

More recently, the installation of denitrification units has also been proposed in RAS in order to decrease water exchange commonly done for nitrate control [50]. Monitoring and removing nitrate in aquaculture systems is further supported by the following facts: (1) environmental regulations usually establish discharge limits that are also applied to aquaculture effluents; (2) by controlling nitrate concentrations, one can prevent high nitrite levels that could otherwise increase due to incomplete denitrification in the water column or sediment; (3) by inducing denitrification, the alkalinity of the water will increase and will balance the alkalinity loss that occurs with nitrification and thus the buffering capacity of the water can be restored if both processes occur; [21].
Available technologies for nitrate removal (denitrification) in recirculation systems commonly rely on heterotrophic microbial metabolism and can include activated sludge systems, fluidised bed reactors, packed bed reactors and moving bed bioreactors [21]. However, as demonstrated in Table 2 from the reviewed literature, the COD content is usually not enough to drive stoichiometrically the denitrification reaction. Thus, similarly to the treatment of secondary effluents, the addition of carbon sources (e.g., methanol) is usually necessary to guarantee ideal C/N ratios for complete denitrification. However, the return of unused organic matter carried over from the denitrification reactor to the aquaculture tanks may cause the undesired growth of bacteria and further consumption of oxygen, thus increasing the risk of generating hypoxic conditions in the fish tank, which can lead to death of the farmed species if no further aeration/oxygenation is provided. The addition of external organic matter can be avoided with the use of autotrophic bio-reactors, using carbon dioxide as carbon source, and H$_2$ as energy source [51], in which hydrogen can be introduced from a compressed cylinder or generated in-situ at the cathode of an electrochemical cell [24, 27]. More recently, it was suggested that bioelectrochemical systems (BES) could be a promising technology for the removal of nitrogen compounds from recirculation systems [52], transferring electrons from an electrode surface (i.e. the cathode electrode) directly to denitrifying microorganisms performing nitrate reduction to dinitrogen. The latter process is referred to as bioelectrochemical denitrification [53] and its application for the treatment of both aquaculture and secondary effluent streams is discussed below.

2.3 Bioelectrochemical Systems (BES)

Bioelectrochemical systems (BESs) are recently being studied as a novel technology for denitrification [54, 55]. These are systems that rely on reactions occurring at inert surfaces of conductive materials (that is, electrodes), in which attached microorganisms perform oxidation/reduction processes as a consequence of the difference in potential between electrode and substrate [56]. As a result of these different potentials, electrons can (1) be transferred from a reduced organic substance to an anode electrode poised at more positive potential, or (2) be taken up from a cathode electrode (at a more negative potential) and delivered to an oxidised substrate. Thus, oxidation and reduction processes are physically separated and, in general, an ion-selective membrane can be used in between the two electrodes, creating two distinct chambers in order to guarantee the
separation of electron donor and acceptor while enabling transport of ions to close the electrical circuitry [57-59]. The theoretical redox potentials (i.e. ability to donate or receive electrons) of some compounds of relevance to this thesis are presented in Table 3.

One common type of BES is the Microbial Fuel Cell (MFC), which aims at power generation. The process is sustained by exergonic reactions such as the oxidation of organics at an anode electrode, coupled to the reduction of substances such as oxygen (with a more positive redox potential) at a cathode electrode [58]. Alternatively, a fine control of electrodes potential or cell voltage in another type of BES (Microbial Electrolysis cell - MEC) enables endergonic reactions to occur, albeit requiring some energy input [58].

Most literature reported so far has mainly focused on anodic processes regarding both MFC and MEC. In this sense, previous studies include a variety of organic compounds as electron donors, as well as microbial community analysis, formation of intermediate compounds and effects of different operational parameters [60-66]. Since more detailed information about cathodic processes are still needed, a short review about the use of BES for nitrogen removal is presented hereafter, with special consideration to the cathodic processes applied for denitrification.

Table 3 – Theoretical redox potentials of relevant compounds to the scope of this thesis: from most positive (likely to gain electrons) to most negative (likely to donate electrons).

<table>
<thead>
<tr>
<th>Redox Couples</th>
<th>E₀ Standard Potential (V)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl₂ / Cl⁻</td>
<td>+1.36</td>
<td>[67]</td>
</tr>
<tr>
<td>N₂O / N₂</td>
<td>+1.36</td>
<td>[68]</td>
</tr>
<tr>
<td>NO / N₂O</td>
<td>+1.18</td>
<td>[68]</td>
</tr>
<tr>
<td>½ O₂ / H₂O</td>
<td>+0.82</td>
<td>[1, 68]</td>
</tr>
<tr>
<td>NO₃⁻ / NO₂⁻</td>
<td>+0.42</td>
<td>[1]</td>
</tr>
<tr>
<td>NO₃⁻ / NH₄⁺</td>
<td>+0.36</td>
<td>[69]</td>
</tr>
<tr>
<td>NO₂⁻ / NO</td>
<td>+0.35</td>
<td>[68]</td>
</tr>
<tr>
<td>SO₄²⁻ / SO₂ + H₂O</td>
<td>+0.17</td>
<td>[67]</td>
</tr>
<tr>
<td>SO₃²⁻ / H₂S</td>
<td>-0.17</td>
<td>[1]</td>
</tr>
<tr>
<td>S₀ / H₂S</td>
<td>-0.28</td>
<td>[1]</td>
</tr>
<tr>
<td>CO₂ / Acetate</td>
<td>-0.28</td>
<td>[1]</td>
</tr>
<tr>
<td>2H⁺ / H₂</td>
<td>-0.42</td>
<td>[1]</td>
</tr>
</tbody>
</table>
2.3.1 Nitrogen removal in BES

Many studies have shown bio- anodic organic matter oxidation coupled to bio-cathodic nitrogen removal and power generation in Microbial Fuel Cells [53, 54, 70-75]. In the study done by Virdis and collaborators [53], the setup was organised as a “loop configuration”, where an acetate- and ammonium-containing stream was inserted in the anodic chamber for acetate reduction, carried to an external aerated compartment for nitrification, and then finally passed through the cathodic compartment for denitrification. Overcoming the need for the additional nitrification step, simultaneous cathodic nitrification and denitrification was evaluated and showed a nitrogen removal efficiency of approximately 94% when in continuous feeding regime [72]. In this study, the authors pointed out that an efficient N removal occurred with the advantages of having a small biomass formation, low energy expenditure and small demand for carbon source compared to an ordinary heterotrophic process. In fact, much research has also been done for the applicability of cathodic reactions in BESs for the removal of nitrate from waters/wastewaters in the presence of only small (or insignificant) amounts of organic matter, such as groundwater [54, 76, 77] and drinking water [78]. As exposed previously for both aquaculture application and for the treatment (polishing) of domestic wastewater, autotrophic denitrification in BES may be an alternative to the addition of organic compounds or to sparging H₂ in the water column, in which electrochemical H₂ generation is possible at a cathode electrode if the cathodic potential is sufficiently low. Since hydrogen is generated in situ, its delivery to a denitrifying biofilm is enhanced, increasing the removal efficiency of nitrogen compounds [18, 79]. Denitrification can also be achieved in BES by transferring electrons directly from an inert conductive surface by applying specific electrical potentials which are higher than the hydrogen generation range [53, 54, 70]. Thus, electrons would be promptly used by denitrifying microorganisms for nitrate reduction, without the need for hydrogen [80].

Therefore, in complete absence of organic electron donors, autotrophic denitrification could play an important role in the removal of the nitrate carried over in the secondary effluent, as well as in the removal of nitrate from aquaculture streams. Overall, a bioelectrochemical autotrophic denitrification is advantageous over traditional heterotrophic denitrification due to less biomass formation (avoiding clogging of the
reactors), and due to the fact that no external organic matter is required in the process, which eliminates the need of supply and on-site storage of chemicals and avoids further carbon contamination into watercourses [21, 22]. In fact, a few studies have demonstrated cathodic denitrification by combining it with an abiotic anode compartment [81-83], in which electrons will be generated abiotically by electrochemical water splitting at a counter electrode (i.e., the anode electrode), with the simultaneous production of oxygen, as exemplified in Figure 5. The electrons generated from the anodic oxygen evolution are transferred to the cathode through an external circuitry where they are used for microbial-mediated denitrification. Interestingly, as previously indicated in Figure 4, aeration/oxygenation of aquaculture fish tanks is extremely important due to requirements for fish respiration [33]. In general, an oxygen concentration of at least 4 mg L$^{-1}$ O$_2$ is required in the fish tanks [84], but concentration requirements might be higher depending on the species of interest and on the fish density stock. Therefore, anodically generated oxygen is also desirable and beneficial for the proposed application.

While a multitude of examples of applications of microbial electrochemical technology to treat nitrate-contaminated water streams are presented in the scientific literature (e.g., wastewater and groundwater) [54, 70, 83, 85], to the best of our knowledge, there are currently no studies demonstrating bioelectrochemical denitrification in seawater streams (which are commonly used in aquaculture), or as a polishing mechanism of denitrification from secondary effluents.
2.3.2 Reduction pathways and electron acceptors in denitrifying cathodic BES

As shown in previous studies, denitrification involves a series of reactions that occur under anoxic condition in the presence of oxidised nitrogen compounds. The cathodic removal of nitrate from water/wastewater can be represented by the half-reactions in Equation 8 to Equation 11 [70]. As it can be observed from the reactions, compounds such as nitric oxide and nitrous oxide can still be formed as intermediates and the release of such gases to the atmosphere – as well as the accumulation of nitrite – may also occur if the overall reaction is not complete. It is important to emphasise that, ideally, the electrons used in those reactions should be provided by the cathode and not by an organic compound, as indicated in Figure 5. Thus, these are the main reactions of interest in this PhD project.

**Equation 8** \( \text{NO}_3^- + 2e^- + 2 \text{H}^+ \rightarrow \text{NO}_2^+ + \text{H}_2\text{O} \)

**Equation 9** \( \text{NO}_2^- + e^- + 2 \text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O} \)

**Equation 10** \( \text{NO} + e^- + \text{H}^+ \rightarrow \frac{1}{2} \text{N}_2\text{O} + \frac{1}{2} \text{H}_2\text{O} \)

**Equation 11** \( \frac{1}{2} \text{N}_2\text{O} + e^- + \text{H}^+ \rightarrow \frac{1}{2} \text{N}_2 + \frac{1}{2} \text{H}_2\text{O} \)
Overall reaction:

Equation 12  
\[ 2 \text{NO}_3^- + 10\text{e}^- + 12\text{H}^+ \rightarrow \text{N}_2 + 6\text{H}_2\text{O} \]

Studies on cathodic denitrification have most commonly reported the reduction of nitrate [27, 54, 70, 71, 77, 78, 81, 86-92], although nitrite and nitrous oxide cathodic reduction are also known to occur [53, 55, 75, 93, 94]. In fact, the formal potential required for nitrate reduction to nitrite (\(E_{\text{o}}^\circ \text{NO}_3^- / \text{NO}_2^- = +0.42 \text{ V vs SHE} \)) is higher than that for Nitrite reduction (\(E_{\text{o}}^\circ \text{NO}_2^-/\text{NO} =+0.350 \text{ V vs SHE} \)). Thus, upon applying a negative cathodic potential, the energy obtained by the bacteria and transferred from the electrode is higher when nitrate is available as electron acceptor [70, 74]. Although it is shown thermodynamically that the use of nitrate over nitrite is preferable, as previously cited, a concomitant consumption of nitrite and nitrate was also previously shown when loading the same N concentrations concomitantly (approx. 50 mg L\(^{-1}\)N as each of the referred compounds) [93]. The authors also observed that the removal rate of nitrate was slightly smaller than that of nitrite. Since the nitrite concentration applied was much higher than the concentrations achieved as intermediate formation during nitrate consumption (i.e. only 5 mg L\(^{-1}\) NO\(_2^-\) -N accumulated in the referred study when nitrate was added as sole electron acceptor), it would be possible that nitrite starts being toxic to the cells and thus it could tend to be consumed in a faster rate even in the presence of nitrate. However, this phenomenon still requires further investigations.

The reduction of \(\text{N}_2\text{O}\) was demonstrated to occur in cathodic biofilms poised at -0.2 V vs SHE, showing that the use of this compound as electron acceptor can sustain biological respiration with energy production in BES [95]. However, generally not all (stoichiometric) intermediate \(\text{N}_2\text{O}\) produced is reduced if \(\text{NO}_3^-\) is still available, and the nitrous oxide tends to accumulate in the cathodic chamber. Studies showed that \(\text{N}_2\text{O}\) produces some residual current that was not related to the presence of \(\text{NO}_3^-\), what confirms that \(\text{N}_2\text{O}\) consumption rate is probably lower than its generation (and consequently lower than \(\text{NO}_3^-\) consumption rate) [75, 96]. Because of that, it was suggested that \(\text{NO}_3^-\) would still be a more preferred electron acceptor than \(\text{N}_2\text{O}\) and the accumulation of this compound can represent up to 10% of electron losses during cathodic denitrification from \(\text{NO}_3^-\) at relatively high applied potential [96]. Contradicting those findings, the theoretical potential for reduction of \(\text{N}_2\text{O}\) (\(E_{\text{o}}^\circ \text{N}_2\text{O}/\text{N}_2 = +1.355 \text{ V vs SHE} \)) is higher than that for nitrate (\(E_{\text{o}}^\circ \text{NO}_3^-/\text{NO}_2^- = +0.433 \text{ V} \))
vs SHE) and thus one would consider the former as a more thermodynamically favourable substrate. However, in practice, bacteria tend to use nitrate more easily, as explained above.

In general, it can be assumed that nitrous oxide is more likely to accumulate in a system than would nitric oxide (NO). In fact, few species such as Corynebacterium nephridii have been previously shown not able to reduce nitrous oxide to nitrogen gas [97, 98]. It is likely that microbial communities enriched with such species may perform incomplete denitrification, leading to the accumulation of N₂O. Furthermore, as reviewed by Richardson et al, the presence of NO is harmful for the cells, which would be killed in case this intermediate cytotoxin is accumulated in the system [5]. This explains why a few studies have neglected the accumulation of NO in denitrifying BES [54, 96].

**Alternative (competitive) pathways during cathodic nitrate reduction**

**Dissimilatory Nitrate Reduction to Ammonium (DNRA)**

Nitrogen cycling in natural environments involves a series of different biological reactions, including aerobic oxidation of ammonium during nitrification (i.e. sequential ammonium and nitrite oxidation to nitrate), followed by the reduction of nitrate to dinitrogen gas (denitrification) in the absence of dissolved oxygen [5, 99]. Another possible but less studied pathway for nitrate reduction is the dissimilatory nitrate reduction to ammonium (DNRA), whereby nitrate is converted directly to ammonium under anoxic conditions [99]. It was previously shown that bacteria able to perform DNRA can be isolated from nitrate-contaminated water and soil [100]. In addition, this phenomenon is also naturally observed in marine and freshwater sediments [101-103], accounting for up to 30% of nitrate reduction processes, thereby slowing down nitrogen removal through denitrification in such systems [103]. The following half reaction describes the process, that has its formal redox potential at approximately +0.36 V vs SHE [69, 104]:

\[
\text{Equation 13} \quad \text{NO}_3^- + 10\text{H}^+ + 8e^- \rightarrow \text{NH}_4^+ + 3\text{H}_2\text{O}
\]

In engineered systems aimed at wastewater treatment, the occurrence of the DNRA pathway can represent an important challenge towards nitrogen removal. As an undesired pathway, it leads to a high percentage of the nitrogen being retained in the water
undergoing denitrification [105], as the regenerated ammonium does not further react in the anoxic treatment stages. Similarly to what was observed in natural environments, DNRA pathway was shown to account for approximately 34% of cathodic nitrate/nitrite reduction in a fundamental study with pure culture of Pseudomonas alcaliphila [106]. Furthermore, ammonium nitrogen was also previously detected in low concentrations in some BES performing denitrification [70, 91, 92, 107]. However, to our best knowledge, DNRA was never quantitatively characterised in a mixed culture denitrifying cathodic biofilm and further investigations about the mechanisms driving this process in BES systems are encouraged.

**Methanogenesis**

Methanogenesis could also be considered a competitive process happening in the cathode chamber of BES, where electrons are consumed for methane formation from CO₂, thus reducing coulombic efficiency of the BES. However, it has been previously shown that processes such as denitrification and DNRA are more favourable and electrons are mainly consumed via those processes than via methanogenesis pathway when nitrate or nitrite are present. Furthermore, nitrite is also shown to be highly toxic to methanogens, inhibiting over 95% of methane production [108], whereas the presence of ammonium is usually also an important factor leading to methanogenesis inhibition during anaerobic digestion [109].

**Presence of Dissolved Oxygen within cathodic area**

The presence of oxygen in atmospheric concentrations seems to have no - or less - inhibition effect to enzymatic activity during assimilation of nitrogen (uptake by cell growth), if compared to the dissimilatory enzymatic activities (nitrification and denitrification) [110]. Some of the enzymes involved in the reduction of N compounds during denitrification are able to use not only NO₃⁻, NO₂⁻ or NO or N₂O, but also O₂ as electron acceptor (facultative anaerobes) [5]. The fact that denitrifying microbes can switch for the use of different electron acceptors has an important impact in the current study, because oxygen is a very good electron acceptor (Eₒ' ½ O₂ / H₂O = +0.82 V vs SHE [1]). As such, in the presence of oxygen, it is likely that denitrification reactions are suppressed and therefore nitrogen removal efficiencies would drop in case aerobic conditions occur.
During simultaneous nitrification and denitrification reactions, cathodic oxygen levels in between 1.97 and 4.35 mg L⁻¹ O₂ still allowed denitrification to occur, showing very low levels of NOx in the effluent [72]. Denitrification reactions in the referred study were strongly dependent on nitrifying microbes on outer layers of the biofilm, and the influence of oxygen in an independent denitrifying biofilm was not evaluated. Similarly, a study showed a two-stage MFC, in which (1) oxygen was the electron acceptor in a first oxic zone (allowing the occurrence of nitrification in the presence of ammonium) and (2) nitrate was the electron acceptor in a second denitrifying (anoxic) zone [88]. Since the setup used in this study included the flow of the oxic effluent towards the anoxic compartment, it is likely that stratification has occurred to some extent within the denitrifying biofilm on the electrodes surface, but this aspect was not evaluated in the referred study.

Nitrate reductases were previously found in smaller amounts when an effluent from an air cathode was used as feed for cathodic denitrification [74], confirming a possible shift from the use of NO₃⁻ to O₂ as electron acceptor. However, in that specific study, it was likely that the presence of both approximately 31 mg L⁻¹ COD (corresponding to 39 mg L⁻¹ sodium acetate) and Oxygen (concentration not informed) could be responsible for a reduction in Coulombic Efficiency observed during that specific feeding regime.

2.3.3 Galvanostatic vs potentiostatic operation modes: the achieved cathodic potentials

When considering the operation of a denitrifying BES as a Microbial fuel Cell (at a fixed external resistance) in which the current generation is highly dependent on exergonic reactions, the cathodic potential is slightly variable and can achieve potentials of -0.242 V vs SHE when removing nitrogen in the cathode compartment [72]. Similarly, although the actual achieved cathodic potential will certainly be configuration dependant, the application of a fixed cell voltage (0.9 V) was previously shown to lead the cathodic potential to reach -0.1 V vs SHE during nitrate reduction [81]. Since those potentials are more positive than the theoretical potential for hydrogen formation, no hydrogen would be formed at the cathode, and electron transfer is likely happening directly from the electrode to the microorganisms performing denitrification.
When considering the operation of a denitrifying BES as a Microbial Electrochemical Cell in which an abiotic anode is used (herein considered ideal if the wastewater lacks enough organic matter as electron donor), either a galvanostatic (current controlled) or potentiostatic (potential controlled) modes can be considered. Previous operation of a BES with fixed currents showed higher nitrate removal rates and N₂O accumulation with 15 mA, if compared to the current of 5 mA [96]. However, Islam and Suidan [111] showed that nitrate removal efficiency increases with current intensity only until a certain extent. They observed that increasing the current to values higher than 25 mA could reduce the nitrate removal possibly due to hydrogen inhibition. However, the achieved specific cathodic potentials were not monitored in those studies.

Although the mode of operation of those referred studies implies slightly variable cathodic potentials, it is observed that the growth and operation of cathodic biofilms is in fact strongly dependent on the effective cathodic potential. In a study focused on an anode-cathodophilic electrode proceeding alternating organic oxidation and nitrate reduction, it was observed that a potential of -0.4 V vs SHE was the most suitable potential for cathodic denitrification [86], whereas a potential of 0 V vs SHE showed only low activity in regards to current production and denitrification rate [86]. Similarly, another study confirmed that the application of more negative cathodic potentials such as -0.2 V vs SHE, if compared to potentials of -0.1 or +0.1 V vs SHE, can lead to higher nitrate consumption and N₂O production/reduction rates, as well as higher current generation [96]. Those studies showed that the required cathodic potential for nitrate reduction under experimental conditions is in fact much more negative than the theoretical one (E°' NO₃⁻ / NO₂⁻ = +0.42 V vs SHE [1]). Thus, cathodic potential has to be low enough to enable electron transfer from the electrode to the cells to proceed with denitrification in a BES [72].

Although electron transfer and nitrate reduction are feasible at relatively high cathodic potentials (i.e. from -0.2 to 0 V vs SHE) in Microbial Fuel Cells aiming energy generation [96], the application of lower (controlled) cathodic potentials was shown to improve denitrification rates in a few studies [96, 112]. In fact, more energy is available to microbes when a bigger difference in between electrode and substrate (electron acceptor) potentials are achieved. Thus, a higher electromotive force will be achieved at lower cathodic potentials [96]. However, although potentially higher energy is gained by the cells for their
metabolism when low cathodic potentials are applied, the quality of the effluent (in terms of intermediate products) may be negatively affected when the cathode is operated at potentials lower than -0.23 V vs SHE [82]. Alternatively, if a specialised microbial community is present at the cathode, the application of more negative cathodic potentials might enable microbial electrosynthesis of hydrogen and/or reduced organic compounds such as acetate [113-115], which could potentially be further used by autotrophic and heterotrophic denitifiers respectively, thus improving nitrate removal rates. In fact, the addition of a previously enriched (electroactive) microbial consortia was already shown to considerably improve current generation and cathodic sulphate reduction rates [116]. Moreover, concomitant heterotrophic and autotrophic denitrification in BES was previously demonstrated by generating hydrogen electrochemically while adding small amount of acetate or methanol as carbon/electron source [18, 78]. Nevertheless, although the application of low (controlled) cathodic potentials may somehow represent an alternative for the improvement of nitrate removal rates via both autotrophic and heterotrophic denitrification, to the best of our knowledge, there are no systematic studies on the effects of operating a mix culture cathodic denitrifying biofilm (including long term operation effects) with potentials lower than -0.7 V vs SHE.

2.3.4 pH and buffer capacity

The maintenance of pH around neutral values is certainly a requirement for the well-functioning of a wastewater treatment system through biological processes. Most organisms grow under those neutral values and only a few species are able to do it at pH lower than 3 or higher than 9. Since Cation Exchange Membranes (CEM) are commonly used in MFC to separate waste streams and increase electron recovery [117], some limitations can arise due to different pH gradients in anodic and cathodic electrolytes [118]. If a molecule of acetate is used as an organic electron donor to an anodic biofilm, its oxidation will produce seven protons, what tends to decrease the pH within the anodic chamber. Contrarily, the reduction of molecules such as nitrate within the cathodic chamber will increase the pH due to consumption of protons (6 mol of protons per mol of nitrate). A reduced anolyte pH can be compensated by the consumption of H⁺ ions at the cathode (increased pH in cathode) [72, 117]. However, since cations such as Na⁺, K⁺, NH₄⁺, Ca²⁺ and Mg²⁺ can also migrate through the membrane, a likely limited proton diffusion will happen, leading to concentration gradients in between cathode and anode
(increased pH in cathode and decreased pH in anode) [53, 59, 117, 119]. The pH gradient in between anodic and cathodic compartments is shown to range from 1.52 to 2.13, depending on the conductivity of the water, which could result in losses of up to 0.125 V, which can correspond to 15 % of energy losses [54]. Therefore, in order to avoid a pH change in biological treatment systems, a buffer solution such as phosphate or bicarbonate is generally used [1, 53, 120]. Although an increase in phosphate buffer is known to improve denitrification performance in BES [121], its addition is generally not recommended in real applications as it may cause further contamination of the water with phosphorus. Thus, the main buffer capacity naturally present in most water/wastewaters is in fact that available from the bicarbonate system, which equilibrium (speciation) equation is demonstrated in Equation 14. During denitrification, protons will be consumed from the acid form H$_2$CO$_3$ transforming it into the bicarbonate HCO$_3^-$ form before a sharp pH rise can occur. Noteworthy, the presence of each carbon form (carbonic acid, bicarbonate or carbonate) is strongly dependent on pH, which will dictate the effective buffer capacity of a water stream depending on the total concentration of inorganic carbon.

\[
\text{Equation 14} \quad \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{CO}_3^{2-}
\]

The use of a loop configuration in which the anode effluent is fed into the cathodic compartment in BES also demonstrated to increase the buffer capacity of the systems, since the acidified anodic stream will be compensated with the cathodic reactions [53]. In addition, it was also previously shown that a shift in the activity of an electrode biofilm can be done in order to catalyse the reduction or the oxidation of the electrode in the presence of acetate or nitrate respectively [86, 122]. Therefore, a same bio electrode was working alternately as either anode (oxidising organics) or cathode (denitrification). Inasmuch as the frequent neutralization of the electrolyte pH leads to a stabilised metabolic activity for both anodic and cathodic functions, the alternating role of the biofilm would avoid changes in pH due to the interruption of acidification or basification commonly observed during standard biofilm operations.

Another method to avoid the pH rise in the cathodic chamber is the use of an acidified anodic compartment, which increases the generation of protons and potentially provides a more effective transport of those protons through the CEM towards the cathodic compartment [123]. Alternatively, many studies have attempted to completely remove the
membrane from the system, which could reduce installation and maintenance costs while preventing sharp pH changes both at anode and cathode areas [124-126]. However, some drawbacks can commonly be observed in this setup, as described in Section 2.3.6.

Furthermore, although those practices are common procedures in fundamental studies of BES, the real application of this technology to the treatment of water streams still faces an important challenge regarding the intrinsic buffer capacity of the wastewater itself. Therefore, the applicability of BES as mechanism for nitrate removal from waters with reduced buffer capacity should be further addressed.

2.3.5 Potential losses and electrolyte conductivity

In general, potential losses can occur in BES due to (1) mass transfer limitation (i.e. inefficient transport of substrate between the bulk solution and the biofilm); (2) limited bacterial kinetics; (3) electron transfer limitation (i.e. inefficient transport of electrons between the biofilm and the electrode) and (4) Ohmic losses (i.e. resistivity of bulk solution, membrane and electrode/electrical connectors material) [58, 119].

Mass transfer limitations are avoided by applying an effective mixing regime within the cathodic and anodic compartments [127], whereas electron transfer losses can be reduced with the use of biological catalysts. Moreover, although charge transfer resistances can be the main responsible for the total internal resistance in young cathodic biofilms, it tends to decrease with increasing biofilm ages [93].

Although the removal of nitrate from artificial wastewaters can be achieved with the use of BES systems [70, 71], the application of this technology to the treatment of waters containing low conductivities faces another challenge [85]. Since conductivity in groundwater, for example, is considerably lower than wastewaters (up to 1000 μS cm⁻¹ and 2000 – 11000 μS cm⁻¹ respectively), one can assume that there are some limitations in applying a BES to nitrate removal under this condition [54]. Because of that, many studies have been done in order to understand and minimise those losses [117-119]. When the water undergoing treatment has a low conductivity, a limited electron and proton transfer due to increased Ohmic resistances can occur, causing a reduction in current production, a lower conversion of NO₃⁻ to N₂ and higher accumulation of intermediates [54]. In the referred study, a linear relationship of nitrogen removal with bulk conductivity in
MFC was observed. In addition, the cathodic potential in the referred MFC was reported to increase from -0.14 V vs SHE to -0.04 vs SHE when the conductivity was increased from 1000 µS cm\(^{-1}\) to 4000 µS cm\(^{-1}\), which confirms bigger losses when facing lower bulk conductivity.

As previously reviewed by Oliot et al., the flux of ions between the electrodes in electrochemical systems is driven by diffusion (activity gradient), migration (electrostatic potential gradient dependent on electrolyte resistivity) and convection (fluid agitation/flow) [128]. However, considering that (1) concentrations of ions in the bulk solution are high enough and (2) minimal concentration gradients are happening at the diffusion layer (interface liquid x biofilm), then the flux of ions through diffusion in BES can be considered negligible. Therefore, in the absence of agitation, the electrolyte conductivity/resistivity will play a key role in the transfer of ions (migration) between cathode and anode.

Although increases in electrolyte conductivity with salt concentration may be an important attempt to reduce ohmic drop and consequently to improve current generation in microbial electrochemical cells, it has been previously shown that power production in microbial fuel cells only increases until a certain extent with addition of NaCl, and can decrease by 50% with 40 mg L\(^{-1}\) NaCl added [129]. In fact, many microorganisms are known to be sensitive to the osmotic pressure at high salinities, and thus the possible gain in current production with increased salinity may be counterbalanced by the inhibitory effect on microbial activity, as previously reviewed by Lacroix et al [130].

Increased Ohmic resistances in MEC imply a higher cell voltage and, consequently, a higher required energy input. As explained above, since ion fluxes are also known to occur via convection due to fluid agitation, providing mixing to the system is generally a valid strategy to improve convection and partially compensate for the low conductivity of the media. In addition, the expected Ohmic losses due to a possible low conductivity of the bulk solution can be minimised by (1) reducing the distance in which the cathodic and anodic electrodes are placed in a BES, (2) adding highly conductive connector materials to be used together with usual carbon electrodes [58] and (3) removing the membrane from the system, as discussed in the following section on BES reactor configurations.
Considering the use of single chamber BES as a denitrifying mechanism either for secondary effluent polishing or for aquaculture applications, there is still a compelling need for a deeper assessment of the real wastewater characteristics such as conductivity and buffer capacity provided by the bicarbonate system, associated with other intrinsic parameters such as reactor configuration and mixing conditions and its effects on nitrate removal and voltage requirements.

2.3.6 Reactor configurations

As exposed above, placing the electrodes at small distances within each other is very important to minimise Ohmic losses in BES, especially when the electrolyte has low conductivity. In fact, several cathodic denitrification studies (either in MFC or MEC operation modes) have used a similar flat, rectangular frame configuration [53, 54, 82, 95, 131, 132]. In this configuration, cathode and anode electrodes are placed in parallel and generally separated by a CEM to enable ion transport (i.e. cations and protons from anode to cathode compartments), while separating cathodic and anodic reactions (Figure 6). This setup is characterised by a big membrane surface area (which dictates the electrode’s projected surface area), and a relatively small electrode depth/electrolyte volume. The graphite granules normally used as both cathode and anode electrode materials tend to occupy a big portion of the reactor’s internal volume, offering also a large effective electrode surface area per liquid volume ratio, thus large area for bacterial attachment, potentially enabling the achievement of high denitrification rates.
Figure 6 – Frame-type BES reactor configuration, including anodic and catodic chambers (and their respective current collectors), separated by a membrane.

Tubular BES reactors with concentric anode and cathode compartments separated by a membrane (i.e. with cathode electrodes placed in either inner or outer portions) were also previously tested for nitrate removal [70, 133]. However, considering either the frames or tubular-type reactors, the presence of the membrane may still impose pH gradients between cathode and anode compartments, resulting in potential losses as discussed in Section 2.3.5, especially if suspended solids are present (and expected to cause membrane fouling over time) [134]. In addition, the small electrode depths (usually in the range of a few centimetres, commonly designed to avoid the pH/potential gradients) may also impose an obstacle for the scaling up of such systems.

Avoiding the pH gradient (and the use of membranes) – which will potentially also reduce capital costs [117] and simplify operation of the cells – many studies have attempted to use a single chamber configuration [77, 89, 92, 135, 136]. However, although single chamber BES were shown to reduce pH gradient and facilitate ion transport between cathode and anode, its use for denitrification applications may result in reduced coulombic efficiency (or electron recovery). Firstly, a problem may arise in the single chamber setup if the anodic reactions are being catalysed by microbes for the oxidation of organic matter. Since organic compounds such as acetate can be a better electron donor than the cathode electrode in a BES (\(E^{o'} \text{ CO}_2/\text{CH}_3\text{COO}^- = -0.290 \text{ V vs SHE}\)), the addition of those compounds straight into cathodic (or single) compartments enables heterotrophic nitrogen reduction within planktonic cells and can negatively affect electron uptake from the
cathode electrode [72]. Secondly, in case of hydrogen producing cathodes, a reduced electron recovery may occur due to hydrogen consumption at the anode [125]. Lastly, in case of abiotically operated anodes in denitrifying BES, reducing denitrification activity may occur due to oxygen intrusion and consumption at the cathode, as discussed in Section 2.3.2.

Alternatively, cylinder-shaped upflow (membraneless) reactors have also been proposed in BES either for (1) simultaneous energy generation and organic matter oxidation (MFC) [134], (2) simultaneous anodic organic matter and cathodic nitrate removal [137], or (3) nitrate removal via cathodic hydrogen formation at fixed currents [138]. This setup was previously expected to feature a more economically feasible and easy to operate process, due to reduced installation and maintenance cost related to the membrane [134]. If a porous material is used – enabling the wastewater to sequentially flow through both cathode and anode electrodes – a facilitated ion transport between electrodes is then expected. Since the liquid flows through a defined pathway (i.e. as the system does not have a complete mixing condition), it becomes more practical to avoid CE losses due to a reduced electrolyte cross-over (i.e. containing O₂). In the study done by Ghafari et al [138], a large cathode electrode (producing hydrogen at fixed currents) was placed at the bottom of a tubular reactor, through which the medium flows firstly, before reaching an anodic zone on the upper portion of the reactor (Figure 7).
Thus, if the wastewater to be treated lacks organic matter, this setup could potentially enable autotrophic denitrification at the lower (cathodic) portion of the reactor, before the wastewater reaches an abiotic (anodic) region producing oxygen from water – therefore avoiding $O_2$ cross-over from anode to cathode in the absence of a membrane.

Although this setup has been previously proposed for hydrogenotrophic denitrification at fixed currents, no studies were found in the literature up to date on the use of such systems at controlled cathodic potentials (i.e. potentially reducing losses due to unused $H_2$) applicable to the treatment of either secondary effluents or aquaculture streams. In addition, when considering upscaling such systems, further studies are necessary in regards to ion transfer mechanisms between cathode and anode electrodes in both freshwater and saltwater streams. Furthermore, aiming at optimising such system (i.e. avoiding the presence of inactive areas in which the achieved cathodic potential is not sufficient to drive nitrate reduction), there is still a compelling need to better understand the potential Ohmic losses across different regions of the reactor and within different depths of the cathode. Moreover, it becomes of fundamental importance to understand how the pH and buffer capacity typical of wastewaters could affect the system’s operation in order to estimate a maximum electrode size and maximum loading rates to be applied.
2.3.7 Electrode Materials

A few studies have demonstrated that the performance of BES can also be improved with the use of more suitable electrode materials and configurations such as three dimensional (3D) electrodes. The 3D electrodes comprise a porous configuration enabling the circulation of the electrolyte through its channels and featuring larger surface area for bacteria attachment than a 2D electrode (i.e. graphite plate). The use of three dimensional electrodes such as granules, foams, brushes and other porous materials was previously shown to enhance current generation and biofilm growth in both anodic and cathodic electrodes [139, 140]. Moreover, although graphite granules have long been used as a 3D electrode material in bioelectrochemical systems [70, 127, 141], recent studies have shown that Reticulated Vitreous Carbon (RVC) can be a promising electrode material due to its very high porosity. Although RVC is known to improve electrochemical (abiotic) current generation for some specific applications [142], a pre-treatment of its surface is necessary in bioelectrochemical systems in order to enable bacterial growth [143]. In fact, studies done on the pre-treatment of RVC electrodes with carbon nanoparticles have demonstrated to improve bacterial attachment, the electron transfer rates and current generation [143, 144]. However, to the best of our knowledge, no studies had been done on the use of such porous electrodes for cathodic nitrate reduction in bioelectrochemical systems.
3 Thesis Overview

As exposed in Chapter 2, there are several advantages of the autotrophic cathodic denitrification potentially achieved with the use of an upflow bioelectrochemical system for the treatment of both secondary (treated) effluents and recirculating aquaculture water streams. Although those water streams likely lack enough buffer capacity to sustain cathodic reactions if nitrate levels are high, they should have sufficient buffer capacity to drive full denitrification in relatively low levels of nitrate. Thus, due to limited expected organic matter availability and small buffer capacity, as well as the low expected nitrate concentrations, both secondary effluents and recirculating aquaculture streams can then be considered of a diluted nature and are subject of study in this Thesis. A description of the previously exposed literature gaps and the research objectives of this work are therefore presented below in Section 3.1. The overall methodology used to pursue the research objectives are then presented in Chapter 4, whereas the specific methodology and research outcomes of each proposed objective are presented in Chapters 5 to 8. Sequentially, Chapter 9 provides a discussion about the future perspectives for the applicability of the upflow BES configuration, whereas Chapter 10 summarizes the main conclusions of this work and outlines the recommendations for future work.

3.1 Research Objectives

3.1.1 Objective 1

*Understanding Dissimilatory Nitrate Reduction to Ammonium as a competitive pathway during cathodic denitrification at low cathodic potential.*

As reviewed above, most BES studies detected negligible amounts of ammonium being formed in the cathodic chamber during denitrification. However, preliminary trials in our lab indicated great ammonium generation at low cathodic potential and the ammonium concentrations tended to change over operation time. Although this phenomenon was not previously described in the literature in regards to mixed culture denitrifying BES, it could significantly influence the quality of treated effluent (both as final effluent discharges into watercourses and as recirculating water stream in aquaculture systems). Therefore, the first objective of this work was to demonstrate, quantify and seek a possible minimisation
of ammonium generation from nitrate in a mixed culture biofilm performing autotrophic denitrification at low cathodic potential.

3.1.2 Objective 2

*Enhancing cathodic denitrification in BES operated at low cathodic potentials by acting on (1) the surface area of the materials and (2) the microbial community.*

Although low cathodic potentials can theoretically increase energy availability for the cells during nitrate reduction and can potentially sustain high current densities when adding highly electroactive (previously enriched) microbial consortia [116], this effect was not yet systematically assessed in the available literature for cathodic denitrification. In addition, studies done on use of 3D RVC as electrode material have demonstrated improved bacterial attachment and current generation compared to flat graphite (2D) electrodes [143, 144]. Therefore, the Objective 2 of this thesis was to investigate and compare the possible enhancement of cathodic denitrification performance in both 2D and 3D electrodes operated at low potential and inoculated with mixed inoculum consisting of (1) denitrifying microorganisms, and (2) a specialised microbial community previously known to generate acetate from CO₂ at a cathode. The aim is to assess the effectiveness of a syntrophic community towards cathodic nitrate reduction.

3.1.3 Objective 3

*Demonstrating and optimising a lab scale easy-to-operate cylinder flow through (membraneless) microbial electrochemical system prototype for nitrate removal from both seawater and freshwater streams.*

As explained in Chapter 2, the use of a membraneless system can avoid pH gradients and the potential losses imposed by the use of ionic membrane and the plug-flow cylinder setup is expected to minimize electron losses due to a reduced electrolyte cross-over between cathode and anode areas. Therefore, the Objective 3 of this PhD aims to demonstrate the feasibility of applying an easy-to-operate plug-flow membraneless BES operated at fixed cathodic potential as a new denitrification concept for diluted streams and to understand the limits of the proposed process. Thus, in order to better assess these issues, Objective 3 was further divided as follows:
Objective 3.1 – *Understanding the proposed prototype as a polishing mechanism for nitrate removal from real secondary (treated) effluents from WWTP*

In this sub-objective, the aim is to demonstrate the applicability of an upflow membraneless system as a polishing mechanism for the treatment of low conductivity (and low buffer capacity) real wastewaters containing also low nitrate concentration and understand ion transfer mechanisms and losses in the system when operated under these conditions.

Objective 3.2 – *Understanding the proposed prototype for nitrate removal from synthetic RAS water representing seawater aquacultures.*

Since nitrate removal from marine (seawater) streams in BES was not previously demonstrated in the literature up to date, this sub-objective aims to provide a proof of concept with the use of synthetic medium firstly. The use of synthetic seawater will enable understanding ion transfer mechanisms and potential Ohmic losses of the system with high conductivity water stream, while assessing the effects of buffer capacity under controlled (set) conditions. Moreover, as part of the proof of concept, this objective aims to provide a detailed assessment of the biofilm community composition.
4 Overall Methodology

This chapter provides a description of the materials and overall research methods used to assess the research objectives presented above. The specific operating conditions of each experiment will be detailed in individual results chapters (Chapters 5, 6, 7 and 8).

4.1 Reactors configurations

Bioelectrochemical reactors used for all the experiments comprised a standard three-electrode setup, in which the cathodic potential was controlled with the use of a Ag/AgCl reference electrode (RE) (saturated KCl, +0.197 V vs SHE). Moreover, the anodic (counter electrode, CE) operated abiotically at all times by oxidating water and generating oxygen.

4.1.1 Bottle reactors (Objectives 1 and 2)

In order to carry out in-depth fundamental studies as required for Research Objectives 1 and 2, a bottle reactor configuration was chosen due to its small size and relative easy operation – compared to the commonly used frame-type reactor – which still enabled the use of a membrane separating cathode and anode compartments, to avoid interferences from the abiotically operated anode.

Bioelectrochemical cells for objectives 1 and 2 were constructed with modified glass bottles (working volume capacity of 0.25 L), as indicated in Figure 8. The main chamber of the reactors hosted the cathodic (nitrate reducing) electrode (CAT), whereas a smaller compartment – an inserted glass tube with 0.008 L volume capacity – housed the Counter (Anode) Electrode for abiotic oxidation of water (producing O2). In order to avoid Oxygen intrusion from the anode towards the cathode compartment, a Cation Exchange Membrane, CEM (Ultrex CMI-7000, Membranes International INC, USA) was used to separate both chambers. A Tedlar gas bag with 3 L volume capacity (SKC, USA) was attached to the headspace of the cathodic chamber to avoid overpressure whenever the reactors were operated in batch mode. The anodic chamber was instead exposed to air, to facilitate Oxygen dispersion.
Figure 8 – Schematic representation of Bioelectrochemical Setup and Feed Reservoir used in experiments of Objectives 1 and 2. (1) Working (Cathode) Electrode inserted into the main chamber, (2) Ag/AgCl Reference Electrode, (3) 8 mL glass (anodic) chamber with an attached Cation Exchange Membrane, (4) Platinum wire counter electrode, (5) gas bag (attached to avoid overpressure due to gas production during batch operation modes only), (6) Stirring bar, (7) Stirring plate, and (8) Pump (in use during continuous flow operation modes only).

Both Working Electrode (WE) and RE were inserted into the main chamber, while the CE consisting of a Pt wire was inserted into the anodic chamber. The electrodes were connected to a multichannel potentiostat (CHI Instruments, USA) for cathodic potential control in Objective 1 and to a multichannel potentiostat/galvanostat (VMP3, Bio-Logic, France) for cathodic potential control in Objective 2 (both controlled at -0.9 V vs SHE).

4.1.2 Upflow reactors (Objective 3)

As identified in Section 3.1.3, in order to investigate the feasibility of applying an easy-to-operate plug-flow membraneless BES operated at fixed cathodic potential for simultaneous nitrate removal and oxygen generation and to understand the limits of the proposed process, the reactors used for the experiments in Objective 3 comprised of
upflow membraneless cylindrical reactors in which graphite granules were used as both anode and cathode material (Figure 9). A graphite rod (National CMG rods 74-5671-00 3/16x 12) was inserted within the granules as a current collector in each electrode. Cathodic granules were placed at a distance from the bottom of the reactor, allowing uniform inflow water distribution. Anodic granules – abiotically operated by splitting water and generating oxygen – were contained in a plastic mesh basket and placed at the upper portion of the reactor. Media recirculation was initially applied within the cathode electrode only, to avoid channelling and mass transfer limitations (electrolyte recirculated from the top portion of the cathode, back to its lower portion at a flow rate of approximately 9 L h⁻¹), except when running specific recirculation test. A reference electrode (RE 0) was inserted on the lower portion of the anode-cathode gap, controlling the cathodic potential at -0.9 V vs. Ag/AgCl (-0.7 V vs SHE), with a VMP3 potentiostat (Bio-Logic, France).

Voltage losses across the liquid phase were measured in different regions of the reactor with the use of a multimeter (Fluke 179 True RMS), through four additional (independent) reference electrodes, placed (RE₁) on top of anode, (RE₂) below anode, (RE₃) just above the cathode bed and (RE₄) below the cathode bed, as indicated in Figure 9.
Figure 9 – BES reactor scheme. RE 0: reference electrode connected to the potentiostat, controlling the system via a three electrode setup; RE 1 – 4: additional reference electrodes 1 – 4 used for potential losses measurements with the use of a multimeter; AN: graphite granules anode electrode; CAT: graphite granules cathode electrode; C1 and C2: recirculation circuits 1 and 2 respectively; SP: liquid sampling port.

Specifications of reactors used for objectives 3.1 (freshwater study) and 3.2 (saltwater study) varied slightly and are detailed in Table 4.

Table 4 – Specifications of Reactors used for freshwater and saltwater experiments of Objectives 3.1 and 3.2.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Objective 3.1</th>
<th>Objective 3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume Capacity (TVC)</td>
<td>2 L</td>
<td>1 L</td>
</tr>
<tr>
<td>Net Anodic Volume (NAV)¹</td>
<td>100 mL</td>
<td>44 mL</td>
</tr>
<tr>
<td>Net Cathodic Volume (NCV)²</td>
<td>160 mL</td>
<td>80 mL</td>
</tr>
<tr>
<td>Gap between cathode and anode</td>
<td>7.5 cm</td>
<td>7.0 cm</td>
</tr>
<tr>
<td>Working electrode projected surface (cross-sectional area)</td>
<td>63.6 cm²</td>
<td>31.3 cm²</td>
</tr>
<tr>
<td>Counter electrode projected surface (cross-sectional area)</td>
<td>28.3 cm²</td>
<td>12.6 cm²</td>
</tr>
<tr>
<td>Counter electrode length</td>
<td>7 cm</td>
<td>7 cm</td>
</tr>
<tr>
<td>Working electrode depth</td>
<td>5 cm</td>
<td>5 cm</td>
</tr>
</tbody>
</table>

¹ – NAV is the liquid (working) volume within anodic granules basket, considering the granules occupy approximately 50% of the total volume within the basket.
² – NCV is the liquid (working) volume within cathodic granules region, considering the granules occupy approximately 50% of the total volume within the cathodic region. Unless stated otherwise, the values of NCV were used in calculations of nitrogen removal rates throughout the thesis.
4.2 Electrode materials and pre-treatment

4.2.1 Carbon cloth working electrodes (Objective 1)

The carbon cloth WE used for experiments in Objective 1 consisted of two pieces of plain carbon cloth (Fuel Cell Store, USA) placed in parallel and totalising 35.2 cm² projected surface area. Current collection was guaranteed with the use of Ti mesh and a Ti wire. The carbon cloth WE was pre-cleaned with Isopropanol (50%) for 4 hours with agitation to remove impurities that could be present on the electrode surface, and then rinsed with abundant reverse osmosis water before being placed in the reactor. The choice of carbon cloth as electrode material for Objective 1 reflects its common application in fundamental studies in the field of Bioelectrochemical system [145] and its wide availability within our facilities.

4.2.2 Graphite plates (Objective 2)

Graphite plates and RVC were chosen as electrode materials used for the experiments in Objective 2, as they typically present the respective 2D and 3D structures, enabling a better comparison of cathodic denitrification performance in those two types of electrodes.

The graphite plates used for the experiments in Objective 2 (Morgan AM&T, Sydney, NSW, Australia), totalling 24.6 cm² projected surface area, were pre-cleaned by immersing the electrodes in 1 M HCl, followed by immersion in 1M NaOH (both overnight), then rinsed in reverse osmosis water in succession. Current collection was guaranteed with the use of Ti wire inserted in a small hole made on the graphite plate. Right before starting up the cathodic denitrifying reactors, all electrode materials tested were plasma treated in nitrogen gas (PDC-002 Harric Plasma Cleaner, New York, USA) for 20 minutes to remove any impurity and improve the hydrophilicity of the electrodes.

4.2.3 Reticulated Vitreous Carbon (Objective 2)

The RVC electrodes used for the experiments in Objective 2 (45 ppi – pores per inch – Duocel, ERG Materials and Aerospace Corporation), measured 1 x 1 x 1 cm, totalling 1 cm² projected surface area. In order to test the RVC electrodes with a cheap surface
treatment method while still enabling the formation of a rough surface for bacterial attachment (which could considerably decrease implementation costs in full scale systems), the RVC electrodes were modified with conductive carbon black (Vulcan XC-72, Cabot Corporation, U.S.) via electrodeposition method. For the modification, a mixture of 540 mg of PVDF 5% w/v in NMP solution and 200 mL acetonitrile was sonicated for 20 minutes (PS-20 Untrasonic Cleaning, Unisonics Australia). 90 mg Vulcan and 1.2 mL triton were added to the solution which was then sonicated for another 20 min. The electrodeposition was carried out in an electrochemical cell comprising a glass beaker (100 mL total capacity) containing a cylindrical Ti mesh which surrounded the RVC electrode to be treated in order to ensure an uniform deposition. The surrounding Ti mesh was connected to the negative terminal of a constant voltage power supply (IPS 2303, ISO-Tech), whereas the RVC electrode was connected to the positive terminal. The bottle was filled with the sonicated solution and each RVC electrode was treated individually in the cell by applying a voltage of 30 V for 7 minutes. While the first electrode was being treated, the remain prepared solution containing the carbon black was kept in the sonicator to avoid sedimentation. After the deposition, the treated RVC electrodes were then rinsed in abundant RO water and dried at 35ºC overnight. Right before starting up the cathodic denitrifying reactors, all electrode materials tested were plasma treated in nitrogen gas (PDC-002 Harric Plasma Cleaner, New York, USA) for 20 minutes to remove any impurity and improve the hydrophilicity of the electrodes.

4.2.4 Graphite granules (Objective 3)

Following preliminary results of Objective 2 with different 2D and 3D electrode materials (as explained in Section 6.2.1), graphite granules (EC-100 Graphite Sales, Inc., USA) were used as both anode and cathode material for experiments in Objective 3. External connection was guaranteed by the use of a graphite rod (National CMG rods 74-5671-00 3/16x 12) in both electrodes. Cathodic granules were placed on a perforated plate whereas the anodic granules were contained in a plastic mesh basket.

4.3 Synthetic media composition

All synthetic media prepared for the experiments were previously sparged with N₂ for 30 minutes to remove oxygen.
4.3.1 Synthetic (highly buffered) medium

The cathodic highly buffered medium used for experiments in objectives 1 and 2 consisted of 6 g L\(^{-1}\) Na\(_2\)HPO\(_4\), 3 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.1 g L\(^{-1}\) MgSO\(_4\) •7H\(_2\)O, 0.015 g L\(^{-1}\) CaCl\(_2\) •2H\(_2\)O, 1 g L\(^{-1}\) NaHCO\(_3\), 20-80 mg L\(^{-1}\) NO\(_3\)-N and trace elements solution as previously described [146]. The cathodic medium was previously autoclaved. The anolyte was composed of 6 g L\(^{-1}\)Na\(_2\)HPO\(_4\) and 3 g L\(^{-1}\) KH\(_2\)PO\(_4\) in the beginning of the experiments, and water evaporation was compensated by adding RO water whenever necessary.

4.4 Inoculum source – enrichment of denitrifying microbial community

A denitrifying microbial community was previously enriched heterotrophically in a fed-batch reactor and used for inoculation of BES reactors, unless stated otherwise at the specific methodology sections. A glass reactor with total volume capacity of 1150 mL was filled with 570 mL synthetic media, containing 6 g L\(^{-1}\) Na\(_2\)HPO\(_4\), 3 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.5 g L\(^{-1}\) NaCl, 0.1 g L\(^{-1}\) NH\(_4\)Cl, 0.1 g L\(^{-1}\) MgSO\(_4\) •7H\(_2\)O, 0.015 g L\(^{-1}\) CaCl\(_2\) •2H\(_2\)O, 1 g L\(^{-1}\) NaHCO\(_3\) and trace elements solution as previously described [146]. The reactor was then inoculated with 30 mL of thickened activated sludge from a WWTP located in South East Queensland, while adding electron acceptor (nitrate) and electron donors to final concentrations of 50 mg L\(^{-1}\) NO\(_3\)-N, 147 mg L\(^{-1}\) sodium acetate, 79 mg L\(^{-1}\) methanol and 109 mg L\(^{-1}\) glucose. During the first three days of enrichment, 50% of media was replaced (on a daily basis) for fresh media with the same composition as described above. After this period, daily additions of electron donors and acceptors (at previously cited concentrations) and weekly partial (50%) media replacement were carried out for approximately 5 weeks. An exception was made for the last week operation, in which no glucose was added and sodium acetate / methanol concentrations were increased to 245 and 114 mg L\(^{-1}\) respectively. After this period, the enriched heterotrophic denitrifying consortia was preserved in 25% glycerol (v/v) and kept at -80°C until their inoculation into the BES reactors. Before inoculation, a portion of the preserved denitrifying culture was then kept overnight at 3°C, before being centrifuged for 10 minutes at 12000 RPM. The supernatant – containing the glycerol – was discarded and the settled material was then re-suspended in buffered medium as described in Section 4.3.1. The process was repeated three times.
4.5 Analytical Methods

Previously to analysis, all liquid samples were filtered through a 0.22µm membrane filter (Millipore Express, USA).

The concentrations of dissolved nitrogen species (NO$_3^-$-N, NO$_2^-$ N and NH$_4^+$-N) were determined using a Lachat QuikChem8000 Flow Injection Analyser (Lachat Instruments, Milwaukee, USA).

Greenhouse gases, GHG (N$_2$O and CH$_4$) were further analysed from liquid samples as described elsewhere [147]. Nitric oxide (NO) generation was assumed to be negligible as previously demonstrated [5, 54, 96].

Dissolved Oxygen (DO) and Cl$_2$ in Objective 3 were measured after the anodic zone of the reactors using a DO probe (SevenGo pro™ dissolved oxygen, Mettler Toledo International) and the Free Chlorine colorimetric method for Cl$_2$ (USEPA DPD method 8021, HACH, USA) respectively.

TOC and TIC were analysed using a High Temperature Combustion (680°C/720°C) method with Platinum beads and Pt mesh as Catalyst and near infrared detector (NIRD) for carbon dioxide (Shimadzu TOC-L CSH Total Organic Carbon Analyser with TNM-L TN unit).

Volatile Fatty Acids (VFA – acetic acid, propionic acid, iso-butyric acid, butyric acid, valeric acid, iso-valeric acid and hexanoic acid) and Alcohols (ethanol, propanol and butanol) were determined by gas chromatography using a flame ionization detector (FID, Agilent Technologies 7890A GC System) and a polar capillary column (DB-FFAP with 30m x 0.53mm x 1.0um) and a deactivated Uniliner for on-column injections (Restek Drilled Uniliner, hole on top, PN 21055) with a phosphoric acid treated glass wool for the analysis.

COD measurements were done using the COD Cell Test, with the Photometric Method (Spectroquant®, Merck Millipore, Germany).
4.6 Calculations

4.6.1 Coulombic efficiency

Coulombic efficiency (CE) of batch tests and continuous flow operations were calculated with the following formula:

\[
\text{CE (\%)} = \left( \frac{5(-\Delta \text{NO}_2^\cdot\text{-N}) - 3(\Delta \text{NO}_3^\cdot\text{-N}) - (\Delta \text{N}_2\text{O}_3^\cdot\text{-N}) + 3(\Delta \text{NH}_4^\cdot\text{-N})]}{M \times Q} \times 100
\]

Where \( M = 14 \text{ mg N/mmol N} \) is the molecular weight of nitrogen; \( V \) (L) is the liquid volume of the cathodic chamber; \( \Delta \text{NO}_2^\cdot\text{-N}, \Delta \text{NO}_3^\cdot\text{-N}, \Delta \text{N}_2\text{O}_3^\cdot\text{-N} \) and \( \Delta \text{NH}_4^\cdot\text{-N} \) (mg N L\(^{-1}\)) are the difference of nitrogen concentrations between (1) the end and the beginning of the batch (for batch tests) or (2) between the outflow and the inflow (for continuous flow modes); \( Q \) (Coulombs) is the cumulative integrated electric charge transferred (1) during the batch or (2) through the duration of the continuous flow experiment (set to 1 HRT); and \( F \) is the Faraday Constant (96485 C/mol e).

Although nitric oxide (NO) is also an intermediate compound during denitrification, it was not included in the above formula. Nitric oxide tends to be consumed upon its formation and the accumulation of this compound can therefore be considered negligible as previously demonstrated [5, 54, 96]. In addition, although approximately 9% of losses during cathodic denitrification were reported to be due to accumulation of N\(_2\)O in batches carried out at -0.2 V vs SHE, the percentage of electrons transferred as current and lost as N\(_2\)O was also shown to considerably decrease with decreasing cathodic potentials [96]. Thus, since the applied potential within the first study (Objective 1) of this thesis (-0.9 V vs SHE) is considerably lower than those reported in the referred study, N\(_2\)O accumulation was also considered negligible in the experiments carried out for the assessment of Research Objective 1. However, as means of comparison, N\(_2\)O values started being measured from Research Objective 2 onwards.

4.6.2 Energy consumption and losses (Objective 3)

Energy consumption was calculated for the Research Objective 3 as described elsewhere [148]. The Cell voltage (\( \Delta E_{\text{cell}} \)) was measured by the potentiostat. Potential losses across
the liquid phase in anodic, gap and cathodic regions were measured as $\Delta E_{an} = RE_1 - RE_2$, $\Delta E_{gap} = RE_2 - RE_3$, $\Delta E_{cat} = RE_3 - RE_4$, where $\Delta E$ (V) is the electrolyte’s Ohmic drop measured between two of the additional reference electrodes (RE). Pseudo-Ohmic resistances ($PR_\Omega$) were then calculated according to Ohms’s law ($PR_\Omega = \Delta E \times I^{-1}$), where $PR$ ($\Omega$) is the electrolyte’s resistance of each region and $I$ is the current (A) flowing through the device. The use of the term pseudo-Ohmic resistance herein acknowledges that the overall resistance across the electrolyte might be lower than Ohmic resistances itself if convection significantly contributes to ion transfer, thus this term represents the total electrolyte resistance.

4.7 Microbial community rRNA sequencing (Objectives 2 and 3.2)

After completion of all experiments for the assessment of the Objective 2, microbial community analysis was done by collecting biofilm samples at different portions of the RVC and graphite plate electrodes. Similarly, after completion of experiments of Objective 3.2, biofilm samples were collected at the lower, mid and upper portions of the cathodic granules. The DNA content of the biomass sample was extracted and analysed as previously described [116].
5 Understanding Dissimilatory Nitrate Reduction to Ammonium as a competitive pathway during cathodic denitrification at low cathodic potential

As discussed in the literature review, Dissimilatory nitrate reduction to ammonium (DNRA) is an undesired pathway occurring simultaneously to denitrification in natural environments as well as engineered systems aimed at biological nitrate reduction/removal. Ammonium formation has previously been detected in cathodic compartments of bioelectrochemical systems performing denitrification, although reported concentrations are generally very low. Therefore, this chapter addresses Research Objective 1, aiming to demonstrate and quantify the occurrence of DNRA from nitrate in a mixed culture denitrifying cathodic biofilm operated at low cathodic potentials. A description of the materials, reactor design, media composition and overall methodology (including calculations) used to assess this research objective were previously described in Chapter 4, and a detailed description of the specific operation procedures done to pursue this objective are presented in Chapter 5.1. Following that, the research outcomes are then presented in Chapter 5.2. The overall conclusions of the study are combined in Chapter 10.

5.1 Specific Experimental Setup

5.1.1 Reactor Inoculation and Operation

A bottle-type reactor was prepared for addressing the Research Objective 1 and was mostly operated under potentiostatic mode, with cathodic medium being continuously mixed with a magnetic stirrer to avoid diffusion limitations, except when performing cyclic voltammetry (CV) experiments. A fixed potential of -0.9 V vs standard hydrogen electrode (SHE) was applied to the cathode electrode unless stated otherwise. A denitrifying microbial community previously grown heterotrophically in a fed-batch reactor was used as inoculum source for the cathodic denitrification experiments. After inoculating the cathode with a small amount of 13 mg as COD of the specified denitrifying culture, the BES reactor was operated in batch mode with no media replacement for 22 days (adaptation period). Exceptionally during this adaptation period (in order to gradually adapt the microbes to autotrophic conditions), periodic additions of nitrate (20 mg L⁻¹ NO₃⁻ -N) as electron acceptor, simultaneously of acetate and methanol as carbon sources, were carried out.
whenever concentrations of nitrate were detected to be lower than 5 mg L\(^{-1}\) NO\(_3^-\)-N, corresponding to days 0 (simultaneously to inoculation), 2, 3, 5, 6, 8 and 10. Carbon sources were added at decreasing concentrations, corresponding to COD/N ratios of approximately 8.2, 5.4, 2.9, 1.8, 1.1, 0.5 and lastly again 0.5, respectively, until day 10 as exposed above. Therefore, organic matter was no longer added to the reactor after day 10. After the adaptation period, the reactor was further operated in sequential batch mode with initial 20 mg L\(^{-1}\) NO\(_3^-\)-N and 50% of the media being replaced weekly, in order to keep lower ammonium concentrations in the reactor and avoid washing out the biomass (enrichment period, days 23 - 89). Finally, in order to guarantee a constant supply of nitrate and removal of by-products while still enabling enrichment of electroactive microbial consortia in the biofilm, the reactor was switched to a continuous feed mode (approximately 0.6 L d\(^{-1}\) flow rate, corresponding to 10 hours HRT) from day 90 onwards, with the same nitrate concentration as previously specified. Cyclic voltammetry (CV) experiments were carried out at different phases of reactor operation in the presence of nitrate, which guaranteed turnover conditions without limiting electron acceptors. CVs were performed without agitation, at a scan rate of 1 mV s\(^{-1}\). The applied potential range was from -1.3 to -0.3 and -1.1 to -0.3 V vs SHE for blank (control) and biofilm operation CVs, respectively.

5.1.2 Reactor Operation during batch tests

A preliminary assessment of the biofilm activity was done at approximately 1 month of operation, during the enrichment phase as specified previously. After the reactor reached a steady current under continuous feed mode, specific batch tests with initial concentration of roughly 20 mg L\(^{-1}\) NO\(_3^-\)-N were then carried out for the assessment of DNRA at 5 and 7 months of operation, to evaluate whether the development stage of the biofilm plays a role on ammonium formation. Prior to each individual test, the feed was interrupted and medium was replaced completely with fresh medium while sparging the reactor with N\(_2\). The reactor was otherwise kept in continuous feed mode between the tests.
5.2 Research Outcomes

5.2.1 DNRA during cathodic biofilm adaptation

The behaviour of the reactor during the start-up (adaptation) period is presented in Figure 10. The current started to increase upon inoculation with denitrifying microbial community (Figure 10 A). The nitrate pulse (20 mg L\(^{-1}\) N) was completely consumed after the first two days of operation and was periodically added in the reactor as indicated in Figure 10 B. Simultaneously to the additions of nitrate, acetic acid and methanol were added at decreasing concentrations as previously detailed in Section 5.1. Both organic compounds were no longer present in the reactor after 17 days. Nitrite was sporadically detected in the reactor during the adaptation period at maximum concentration of approximately 2.4 mg L\(^{-1}\), however this compound was consumed afterwards and there was no nitrite left in the reactor at the end of the adaptation period (Figure 10 C). The figure also shows that ammonium starts forming approximately after two days of operation and accumulates to a concentration of 27 mg L\(^{-1}\) in 22 days. The formation of ammonium during this stage could primarily be linked to the presence of acetate and methanol in relatively high concentrations during initial stages of the adaptation phase. As it has been previously shown, high C/N ratios (which can be translated as the ratio of electron donors to acceptors) due to high organic matter concentrations favours the DNRA pathway during heterotrophic nitrate reduction \[149\]. However, the observation that ammonium formation/accumulation still occurs in the absence of organic electron donors after day 17 indicates that DNRA is possible also in autotrophic systems with electrons provided from the cathode.

The DNRA pathway is generally not considered in most cathodic denitrification studies due to the absence or very low concentrations of ammonium usually detected within the denitrifying cathodes \[82, 96\]. However, our results indicate that it is in fact happening in mixed culture denitrifying cathodes, which corroborates a previous publication that reported detection of ammonium in denitrifying microbial fuel cell (MFC) inoculated with non-adapted microbial community \[70\]. Therefore, as nitrate is partially converted to ammonium, the corresponding amount of nitrogen cannot be removed from the solution under the anoxic conditions encountered in this system.
It has previously been reviewed that DNRA can be favoured over denitrification when more reducing environmental conditions occur [150]. A study done with pure culture of *Pseudomonas alcaliphila* showed that proportionally more electrons are transferred from nitrate to ammonium when applying a lower potential such as -0.9 V, as opposed to +0.1 or -0.1 V vs SHE [151]. Although the effects of cathodic potential on ammonium formation were not evaluated herein, those previously reported results give an insight on the reasons why some previously studied BES operating in MFC mode (with much higher cathodic potential) did not detect any ammonium formation [96], whereas up to 4.1 mg L$^{-1}$ ammonium accumulated at the end of preliminary batches in the present study (data not shown).

![Graphs](image)

**Figure 10** – Inoculation/adaptation period (22 days). (A) Current profile; (B) NO$_3^-$ -N profile and (C) NO$_2^-$ -N and NH$_4^+$ -N concentrations, respectively. Single black arrow at day zero indicate inoculation time whereas red arrows between days 0 and 10 indicate additions of nitrate simultaneously to decreasing concentrations of organic matter (acetic acid and methanol). Asterisks indicate additions of nitrate only.
5.2.2 DNRA is linked to biofilm development stage and electron donor availability

Cyclic voltammetry performed before inoculation of the reactor show a lack of significant reductive current at -0.9 V vs SHE, indicating absence or very low catalytic formation of hydrogen under abiotic conditions at this potential (Figure 11). The onset of cathodic nitrate reduction shifted towards more positive potential values as the denitrification activity developed, as shown in Figure 11.

![Cyclic voltammograms performed in turnover conditions at different times of cathodic biofilm development (0, 1 and 5 months). Scan rate 1 mV s⁻¹. The inset indicates onsets of catalytic currents.](image)

Current and nitrogen species time profiles during the batch tests performed at different operational stages are shown in Figure 12. The current increased over time when batches were carried out in the young biofilm (Figure 12 B). However, the current did not seem to be affected by decreasing nitrate concentrations. Furthermore, the current tended to be more stable and higher in magnitude for the batches carried out with the 5 and 7 months old biofilm (Figure 12 D and F). Noteworthy, the applied cathodic potential of -0.9 V vs SHE is lower than the theoretical hydrogen evolution potential (-0.41 V vs SHE), hence hydrogen production is expected. Several studies have previously shown hydrogen formation at cathodic surfaces in bioelectrochemical systems [125, 152], and its production was also shown to be enhanced over time with the use of microorganisms as catalysts when applying the potential of -0.75 V vs SHE [115]. In addition, electrons delivered through a biocatalysed cathode were also previously shown to be completely recovered as hydrogen (100% cathodic hydrogen efficiency) if a negligible diffusion is occurring through
the membrane [125]. Thus, although hydrogen measurements of liquid and gas phases were not done in this work, the current behaviour presented herein corroborates an increasing H\textsubscript{2} formation over time as reported in the above cited literature, which is further supported by the CVs shown in Figure 11 [115].

Figure 12 – Nitrogen species and current profile during batch tests at -0.9 V vs SHE carried out with initial concentration of 20 mg L\textsuperscript{-1} NO\textsubscript{3}\textsuperscript{-}N at different biofilm development stages. (A and B) 1 month old biofilm tests (n=2); (C and D) 5 month old biofilm (n=2); (E and F) 7 month old biofilm (n=3). Nitrogen concentrations are plotted as averages (± standard deviations), whereas current profiles are plotted for each individual test (T1, T2 and/or T3, depending whether they were done in duplicates or triplicates).

Reduction of nitrate from 20 mg L\textsuperscript{-1} NO\textsubscript{3}\textsuperscript{-}N to approximately 8 mg L\textsuperscript{-1} required 2 days of operation when the biofilm was 1 month old, whereas approximately the same amount of nitrogen was reduced within 12 hours during the batch tests carried out at 5 and 7 months of biofilm operation. Nitrite was detected in low concentrations during the 1 month tests and was found to be below detection limits at the end of those batches and also at all times during the batches carried out in months 5 and 7. Furthermore, the amount of ammonium formed during batches at different biofilm ages decreased from 9.6 ± 3.5 to 3.80 ± 0.3 and 0.76 ± 0.4 mg L\textsuperscript{-1}, respectively at 1, 5 and 7 months of operation.
A more detailed analysis of batches carried out at different times of operation indicates a metabolic shift occurring within the biofilm, as shown in Figure 13. As it can be noticed in the bar chart, the percentage of nitrate converted to ammonium tended to decrease over time of biofilm operation/adaptation. An average of 47.8% ± 19.7% of all reduced NO$_3^-$-N was converted to ammonium within the young biofilm, whereas only 5.8% ± 2.8% was converted via DNRA pathway within the 7 month old biofilm. This fact demonstrates that a long time period is required for the denitrifying cathodic biofilm development. A 46 days start-up time was previously reported for a cathodic biofilm [95] and a long maturation time of months instead of weeks was also observed in a study evaluating differences of electrochemical impedance over time for anodic biofilms inoculated with non-adapted biomass [119].

The previously reported fact that more reductive environmental conditions play a role on the occurrence of DNRA helps understanding the higher than usual ammonium formation in this study. However, as the potential applied herein was constant over the whole study period, this hypothesis does not explain the decreasing ammonium formation observed over time. It was previously shown that a high C/N ratio (translated to electron donor/acceptor ratio in autotrophic conditions) stimulated the enrichment of DNRA bacteria, that were able to convert up to 90% of all nitrate into ammonium in a chemostat [149]. In addition, the electron donor/acceptor ratio was in fact found to play a role alongside environmental conditions in DNRA regulation [150]. Furthermore, the effect of
electron donor-to-acceptor ratio was also confirmed in BES pure culture studies [106, 151]. An assessment of Coulombic efficiencies (CE) in the present study indicated that lower efficiencies of approximately 55.4% ± 1.1% were obtained at an early stage of operation, as opposed to Coulombic efficiencies higher than 90% at later stages of biofilm development (Figure 13). A low Coulombic efficiency implies an excess of electrons being transferred from the cathode relatively to those used in the reduction of nitrate, and therefore an excess of free hydrogen was likely to have occurred at early stages. When assuming that (1) the excess electrons delivered from the cathode must necessarily go into hydrogen generation [115, 153] as explained previously, and (2) 1 mmol H₂ requires 2 mmol electrons transferred from the cathode, then an averaged hydrogen production can be calculated from the charge transferred during the batches to be 0.021 (±0.001) and 0.055 (±0.002) mmol H₂ per hour for 1 and 7 months operation, which shows that hydrogen formation was actually considerably smaller in the first month compared to latter stages. However, an important factor for consideration is the ability of the biofilm to consume electrons for denitrification. Since the biofilm was still not fully developed at 1 month operation, its ability to carry out autotrophic denitrification and consume that hydrogen was very poor as indicated by the low nitrate reduction rate (5.1 ±0.3 g N m⁻³NCV d⁻¹ ± SD). Therefore, it resulted in free H₂ within the biofilm which translated into low CE. On the other hand, although calculated H₂ production was higher at latter stages, as explained above, nitrate reduction rates were considerably faster (26.0 ±1.4 g N m⁻³NCV d⁻¹ ± SD). Therefore, this relation between electrons delivered as H₂ and consumed during nitrate reduction was reflected in the higher CE obtained at later stages of operation, and suggesting that the shortage of electrons available as free hydrogen led to a restriction in the DNRA pathway in the 7th month operation. The high electron donor (hydrogen)-to-nitrate ratio established at low CE would have led to DNRA as preferential pathway. Noteworthy, the complete denitrification pathway from nitrate to nitrogen gas requires only 5 electrons for the reduction of each nitrate molecule, whereas the DNRA pathway requires a total of 8 electrons. Therefore, it is understandable that a bigger availability of electrons in the system would enable the formation of ammonium in detriment of the denitrification pathway.

“Since the inoculum used in this study had been originally enriched in the presence of organic matter (i.e. acetate, methanol and glucose), it is likely that there was a shift in
Community structure over time of reactor operation (i.e. enrichment of hydrogenotrophic denitrifiers). This hypothesis supports the observation that electron uptake capacity has increased over time in this study. Thus, an in depth assessment of microbial community structure and its evolution over time is certainly suggested for future work, in order to better understand this phenomenon"
6 Understanding cathodic denitrification in BES operated at low cathodic potentials: is high rate denitrification possible in 2D and 3D electrodes enriched with electroactive microorganisms?

Achieving high removal rates in BES is of fundamental importance when designing and scaling up a treatment unit, as it allows for reduction of installation and operation costs as well as the development of a more competitive technology. In order to assess this issue and potentially achieve high denitrification rates, as previously described for Research Objective 2, two electrode materials were inoculated with enriched (electroactive) microorganisms and tested at -0.9 V vs SHE: a 2D graphite plate and a novel 3D Reticulated Vitreous Carbon (RVC) - modified with electro-deposited Vulcan carbon particles. A description of the materials, reactors design, media composition and overall methodology used to assess this research objective were previously described in Chapter 4, and a detailed description of the specific operation procedures done to pursue this objective are presented in Chapter 6.1. Following that, the research outcomes are then presented in Chapter 6.2, and the main conclusions of the study are combined in Chapter 10.

6.1 Specific Experimental Setup

6.1.1 Electrodes surface area and nitrogen removal rates – considerations for comparison

BES reactors commonly comprise a frame-type setup in which cathode and anode compartments are separated by a cation exchange membrane (CEM). This setup generally features small ratio of electrolyte volume to membrane surface area as well as a short distance between cathode and anode electrodes, as described elsewhere [54, 154, 155]. Commonly used graphite granules (or other 3D material) as either cathode or anode electrodes will partially occupy the volume of cathode or anode chambers, which decreases the electrolyte volume within the reactor and enable a large electrode surface
area to be available for bacteria attachment. Ideally, in a 3D system such as described, the electrolyte easily flows through the channels between the granules, facilitating mass and charge transport within each chamber. The performances of experiments done in such configuration are generally reported as current density, based on the projected surface area of the electrode (which corresponds to the membrane surface area separating the two chambers). In this scenario, only a small portion of the 3D electrode’s surface generally touches the reactors walls, thus the inactive surface area is minimal compared to that of the total active surface (in contact with the electrolyte) available for biofilm growth.

On the contrary, if a 2D (flat) electrode, such as a graphite plate, is used in a frame reactor, and placed parallel to the membrane, the charge/mass transfer between the electrodes and through the membrane (and thus current generation) will be significantly affected if one of the sides of the electrode is touching the reactor wall opposite to the membrane. In that case, only the sides of the electrode that are directly interfacing the electrolyte contribute to the current. In this work, since a bottle type reactor – instead of a frame type – is used for the experiments, all sides of the graphite plates are in contact with the electrolyte, hence they play a role on current generation. Hence, differently from the 3D electrodes, current density calculations of a 2D electrode (based on projected surface area) have to consider the total electrode’s surface area and not only the membrane area. Therefore, the projected surface area of a 3D electrode such as RVC with dimensions of 1.0 x 1.0 x 1.0 cm is herein considered equal to 1cm$^2$, whereas the projected surface area of a same-sized 2D electrode such as graphite plate is the sum of all its exposed projected surfaces (6 cm$^2$).

Furthermore, although nitrate removal rates in BES are most often reported as volumetric rates (g N m$^{-3}$d$^{-1}$), providing an important information in an engineering point of view, reporting the performance of the reactors in terms of the geometric rates (g N cm$^{-2}$ d$^{-1}$, based on electrodes surface area) enable a better comparison of different electrode materials.
6.1.2 Experimental design and inoculation

The Vulcan-treated RVC and the graphite plate electrodes were tested in duplicates by placing each electrode in an independent reactor, hereafter named PL1 and PL2 (containing graphite plate electrodes) and RVC1 and RVC2 (containing Vulcan-modified RVC electrodes). As previously mentioned, the presence of an already adapted (electroactive) acetogenic microbial consortium is expected to improve denitrification performance through syntrophy. Thus, the two reactors containing graphite plate electrodes (which were previously producing H₂ and acetate from electrons and CO₂) [114] were switched from their original operation towards denitrification operation. Bulk liquid samples from those reactors were collected and kept in the fridge for inoculation of the RVC reactors. The plate reactors thus contained an already formed biofilm able to perform electrosynthesis and were further inoculated with 13 mg as COD of a denitrifying microbial consortia grown heterotrophically in a fed-batch reactor (as described in Section 4.4). The RVC reactors RVC1 and RVC2 were then simultaneously inoculated with both the same denitrifying microbial community (13 mg as COD) and the bulk electrolyte sample previously collected from the reactors PL1 and PL2, containing the enriched electroactive microbial consortia.

In order to confirm whether the presence of the previously adapted electroactive microbial community was in fact enhancing electron uptake by the biofilm and participating in denitrification reactions, another reactor (PL3) was inoculated with denitrifying biomass only. A matrix of the experimental setup can be found in Table 5.

Table 5 – Matrix of experimental setup including electrode materials tested and form of inoculation.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Electrode Material</th>
<th>Projected surface area</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL1</td>
<td>Plate</td>
<td>24.6 cm²</td>
<td>AcB (pre-grown) + DEN_In</td>
</tr>
<tr>
<td>PL2</td>
<td>Plate</td>
<td>24.6 cm²</td>
<td>AcB (pre-grown) + DEN_In</td>
</tr>
<tr>
<td>PL3</td>
<td>Plate</td>
<td>24.5 cm²</td>
<td>DEN_In</td>
</tr>
<tr>
<td>RVC1</td>
<td>RVC</td>
<td>1 cm²</td>
<td>DEN_In + AcP</td>
</tr>
<tr>
<td>RVC2</td>
<td>RVC</td>
<td>1 cm²</td>
<td>DEN_In + AcP</td>
</tr>
</tbody>
</table>

*AcP – Electro-Active inoculum from planktonic cells
*AcB – Electro-Active inoculum Biofilm attached to plate electrodes
*DEN_In – Denitrifying inoculum
The catolyte was continuously mixed with a magnetic stirrer to avoid diffusion limitations, except when performing cyclic voltammetry (CV) experiments. All reactors were kept at 35°C inside an incubator at all times. Upon inoculation, the reactors were operated in batch mode for 2-3 weeks without media replacement, in order to avoid wash out of cells and enable biofilm attachment/formation. During this stage, nitrate was being added upon its depletion in each reactor, at concentrations of 60 to 80 mg L⁻¹, depending on each reactor’s requirements. In order to avoid nitrate limitations and guarantee removal of by-products while still enabling enrichment of electroactive microbial consortia in the biofilm, the reactors were switched to continuous feed mode (approximately 0.6 L d⁻¹ flow rate), with the same nitrate concentrations. It is noteworthy that, although this PhD thesis focuses on nitrate removal from diluted streams, high nitrate concentrations were herein used merely to avoid biofilm starvation and guarantee substrate availability during this fundamental-level study phase – which could interfere on the maximum detectable denitrification rates – as it had been observed during preliminary laboratory experiments. The preliminary continuous feed operation was kept for a week, after which a sampling period of 35 days was carried out.

6.2 Research Outcomes

6.2.1 Performance of different electrode materials at low cathodic potential

Figure 14 indicates the cumulative charge (mmol e⁻ cm⁻² projected surface area) transferred from the RVC and plate electrodes during the 35 days sampling period. The results indicate that the total charge transferred from the electrode to the biofilm – calculated based on electrode’s projected surface area – was considerably higher on the RVC electrodes (78.6 ± 5.3 mmol e⁻ cm⁻²) than the plates (17.6 ± 4.9 mmol e⁻ cm⁻²).
Nitrate reduction rates (based on complete denitrification) were $1.1 \pm 0.1 \,(n=4)$ and $1.0 \pm 0.3 \,(n=3) \, \text{mg N cm}^{-2} \, \text{d}^{-1}$ for plate reactors PL1 and PL2 respectively, and $3.3 \pm0.4 \,(n=3)$ and $1.6 \pm 1.5 \,(n=3) \, \text{mg N cm}^{-2} \, \text{d}^{-1}$ for RVC reactors RVC1 and RVC2 respectively (Table 6). Those removal rates are similar to previously reported values for carbon cloth electrodes operating at considerably higher cathodic potential [145], which may indicate the reactors were not operating at an optimised condition herein. In fact, the high variability in the concentration of ammonium (produced through the competitive dissimilatory nitrate reduction to ammonium – DNRA – pathway) and nitrite (denitrification intermediate), indicate that the reactors did not reach a steady state condition (Table 6). The variability of ammonium and nitrite (which contributed to the also variable denitrification rates of RVC2) could be partly attributed to a few episodes of membrane breakage (and consequently oxygen intrusion), which tended to upset the microorganisms and affect the overall nitrate reduction performance.

A relatively high coulombic efficiency of plate reactors PL1 and PL2 (82.1 ±5.4 and 73.1 ±16.2 % respectively) indicated only a small portion of the electrons transferred as current were not used for nitrate reduction reactions, and were likely lost as hydrogen in the plate reactors. On the contrary, the low coulombic efficiency of the RVC electrodes RVC1 and RVC2 (39.0 ±2.8 and 37.9 ±18.8 % respectively) indicate big electron losses (likely as hydrogen) in those reactors.

Figure 14 – Cumulative charge transferred electrochemically relative to projected surface area of cathodic electrodes (Vulcan-treated RVC and graphite plate) tested.
Table 6 – Overall performance of the Plate and RVC reactors during the 35 days sampling period. Except where indicated for total surface area current density, all values are based on projected surface area.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Current Density Projected</th>
<th>DEN rate</th>
<th>NH$_4^+$ loss</th>
<th>NO$_2^-$</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Projected A m$^{-2}$</td>
<td>Total A m$^{-2}$</td>
<td>mg N cm$^{-2}$ d$^{-1}$</td>
<td>% of reduced nitrate</td>
<td>% of reduced nitrate</td>
</tr>
<tr>
<td>PL1</td>
<td>5.2 ±0.8</td>
<td>5.2 ±0.8</td>
<td>1.0 ±0.1</td>
<td>0.2 ±0.2</td>
<td>2.8 ±3.6</td>
</tr>
<tr>
<td>PL2</td>
<td>6.8 ±2.8</td>
<td>6.8 ±2.8</td>
<td>1.0 ±0.3</td>
<td>8.1 ±10.4</td>
<td>6.0 ±10.0</td>
</tr>
<tr>
<td>RVC1</td>
<td>29.4 ±3.0</td>
<td>1.1 ±0.1</td>
<td>3.3 ±0.4</td>
<td>3.5 ±4.3</td>
<td>1.5 ±1.7</td>
</tr>
<tr>
<td>RVC2</td>
<td>22.7 ±3.6</td>
<td>0.9 ±0.1</td>
<td>1.6 ±1.5</td>
<td>17.2 ±17.5</td>
<td>38.9 ±28.1</td>
</tr>
</tbody>
</table>

As expected at low applied cathodic potentials [54, 96], the analysis of additional liquid samples taken after the 35 days sampling period confirmed negligible nitrous oxide accumulation (which accounted for less than 0.3% of all nitrate reduced), except for the reactor RVC1 (5.4 ±0.5%, n=3) of nitrogen accumulated as N$_2$O). Curiously, this was the only reactor which also generated methane (corresponding to an electron loss of 12.9 ±1.2 %), and certainly contributed to the low coulombic efficiency.

The use of bare RVC cathodes in previous BES studies did not result in either bacterial growth or the occurrence of desired electrosynthesis reaction, indicating that a deposited structure of carbon nanotubes (obtained in a preliminary treatment of the electrode) led to the formation of a rough surface, which was in fact the main factor responsible for increased electroactivity of the RVC electrodes [143]. Although the pre-treatment of RVC electrodes with Vulcan particles herein also enabled bacterial attachment/biofilm growth and electrochemical activity, the current densities obtained (29.4 ± 3.0 and 22.7 ± 3.6 A m$^{-2}$ for RVC reactors 1 and 2 respectively) are smaller than that achieved in a previous study on bioelectrosyntesis (150 A m$^{-2}$) [114]. However, a few considerations can be made in regards this issue. Firstly, although the same 45 ppi RVC structure was used in both studies, the type of treatment done on our RVC electrodes is different (Vulcan deposition instead of nanotubes in the previous study), which could have influenced the obtained performance. Secondly, the target product of the bioelectrochemical reactions in the cited
The study was acetate, which dissolves in the liquid phase, whereas the final product of the denitrification reactions herein is a gas (dinitrogen). Thus, although it was expected that the presence of the previously enriched electroactive microbial community could lead to hydrogen and acetate production which could be further utilised by denitrifiers (thus improving current density), it is very likely that bubble formation throughout the RVC electrode may have caused blockage of the electrode/biofilm active sites, preventing mass and charge transport to/from the biofilm. This hypothesis is supported by the fact that bubbles were accumulated on the outer layers of the RVC, as well as on the inner layers (which was observed upon biomass sampling by cutting the electrode), as indicated in Figure 15. Lastly, it is also possible that mass and charge transfer limitation might have occurred due to the formation of a very thick biofilm on the electrode surface. This phenomenon was previously reported for anodic biofilms grown on porous material, even though the pores were of more than 100 micrometres big on the referred study [139]. Moreover, the collection of biomass sample for community structure assessment from our Vulcan-treated RVC electrodes revealed different colour patterns of the biofilm grown on inner and outer portions (Figure 15), which corroborates this hypothesis.
Figure 15 – Electrodes RVC1 (A) and RVC2 (B) before biomass sampling; Electrodes RVC1 (C) and RVC2 (D) after biomass extraction, showing bubbles formed throughout the electrodes during sampling period.

Moreover, although the 11.9 mM carbon provided by the added sodium bicarbonate was enough to produce theoretically 350 mg L$^{-1}$ acetic acid and drive a current of approximately 10.8 A m$^{-2}$ on the plate reactors, it was observed that current generation tended to decrease slightly over operation time. A further assessment of the reactors done after the 35 days sampling period (which was done to include N$_2$O and CH$_4$ measurements) confirmed the decreased activity (data not shown). Thus, in order to assess this issue, the plate reactors were further monitored for a longer period of time. The plate electrodes were chosen for this experiment due to the flat/solid structure of the graphite plates, which enables an easy manual removal of the biofilm from the electrode’s surface without compromising the electrode material. Thus, the electrodes were further operated for approximately 200 days. During this period, current generation as well as nitrate reduction rate decreased constantly as indicated in Figure 16. At day 197, the electrodes were temporarily removed from the reactor and the thick layer of the biofilm was gently removed with a flat tweezers. The electrode was then re-inserted in the reactor and operated for another week. This procedure showed an instantaneous increase in current generation and nitrate reduction activity. Both duplicate reactors responded very similarly to the
procedure, which confirms that the thick biofilm was preventing (1) mass transfer from the liquid phase towards inner portions of the biofilm and/or (2) electron uptake from the electrode towards outer cells. Since this phenomenon had occurred in the flat structure of the plate electrode, it is assumed that it would then certainly lead to great clogging of the fine pores of the 45 ppi RVC structure used herein, from which biofilm removal would become impractical.

![Graph showing denitrification activity](image)

**Figure 16** – Long term operation of a graphite plate biocathode performing denitrification, indicating decreasing activity over time, followed by activity recovery after excess biomass removal (red arrow).

One reason for the excessive bacterial growth observed here may rely on the different theoretical redox potentials required for the reactions of interest in both studies. Hydrogen and acetate formation have a much more negative theoretical redox potential requirement (-0.42 and -0.28 V vs SHE respectively) than that required for denitrification (+0.42 V) [1]. This means that, when attempting to generate acetate bioelectrochemically, applied cathodic potentials should be lower than -0.28 V. Although this acetate produced in the reactor could be further used as electron/carbon donor for heterotrophic denitrification, the very low potential applied is likely also providing great energy for the reduction of nitrate, thus possibly enabling the denitrifiers to outcompete acetogens. Interestingly, a recent study on cathodic denitrification showed that nitrate reduction rates improved with decreasing potentials only until reaching -0.4 V vs SHE [82], which indicates the biofilm on the referred study had reached its maximum denitrification capacity at potentials which would in practice still not enable acetate or hydrogen production. In fact, as indicated in
Figure 17, an applied cathodic potential of approximately -0.45 V vs SHE was required to show an onset of hydrogen formation in our biofilm-containing plate electrodes. Although the verification of acetate production within the reactors could help understanding the denitrification performances and dynamics herein, any acetate eventually produced was in fact expected to be promptly consumed by the denitrifiers within the biofilm. Therefore, acetate measurements were not performed in this study.

Therefore, although promising results were obtained in a previous study with the use of RVC as cathode electrodes, our results show that an excessive biofilm growth is possible during cathodic denitrification at low potentials. Thus, the 45 ppi RVC used in the experiments herein might not be the best option for the proposed denitrification reactions, at least at the tested operational conditions as in the present study.

6.2.2 Effect of inoculation on overall reactors performances

In order to further assess the role of inoculum source on the performance of the reactors and evaluate whether denitrifiers might be out-competing the electroactive acetogens, a new reactor was started with a bare plate electrode as cathodic material, and inoculated only with denitrifying biomass. In order to match the total operation time of the previous
two plate reactors (including the preliminary acetogenic operation before inoculation with denitrifiers), the new reactor was then operated for approximately 100 days before detailed characterization. The assessment of the 35 days sampling period indicated no significant difference in current generation and charge transfer (P>0.05) of the new reactor compared to those reactors that were initially inoculated with specialised (electroactive) acetogens community (Figure 18). The average current density at time of liquid sampling was $7.4 \pm 1.5$ A m$^{-2}$ and denitrification rate was $1.4 \pm 0.4$ mg N cm$^{-2}$ d$^{-1}$ (n=4), indicating also no significant difference in activity to the remain plate electrodes (P>0.05). Those results corroborate with the previous discussion and the hypothesis that the electroactive community might not be playing an important role in the reactor’s activity and this effect is further discussed in the next section on microbial community structure.

![Figure 18](image)

**Figure 18 – Cumulative charge transferred per projected electrode surface area on cloth (a) and plate (b) electrodes.**

### 6.2.3 Community structure assessment

As indicated in Figure 19, through the Principal Component Analysis (PCA), the electroactive planktonic and biofilm inocula used in the experiments (AcP and AcB respectively) were very closely related to each other, whereas the denitrifying inoculum (DEN_IN) differed considerably. After the inoculation and operation of the reactors as demonstrated above, community composition has shifted significantly from those used as inoculum sources, both denitrifiers (DEN_IN), planktonic and biofilm electroactive (AcP and AcB) inoculum types, indicating that a specialised community structure has developed.
enabling the desired nitrate reduction. Interestingly, even though cathode electrodes from reactors PL1 and PL2 were previously performing acetate production and contained high abundance of *Natranaerobium*, *Hydrogenophaga*, *Acetoanaerobium*, and Methanobacteriales [114], the presence of these organisms was only detected in minimum numbers in any of the duplicate plate reactors herein. These results corroborate the similar current densities of plate reactors PL1, PL2 and PL3, even though PL3 did not receive any electroactive inoculum. These observations confirm the reactions of nitrate reduction in the present study did not depend (or depend to a minimum extent) on the previously-adapted inoculum used. Moreover, no significantly different community composition was observed between the reactors, despite the material used and the time of operation (which was shorter for the RVC reactors).

![Figure 19 – PCA analysis of microbial community and cathodic electrode materials indicating diversity of conditions between reactors. AcP – Electro-Active inoculum from Planktonic cells; AcB – Electro-Active inoculum biofilm attached to plate electrodes.](image)

As indicated in Figure 20, a high abundance of OTUs belonging to the genus *Paracoccus* was found in reactors RVC1, PL1, PL2 and PL3 (48, 10, 29 and 27% abundance respectively) and could be regarded as the main responsible for denitrification on the biofilms formed at the electrodes RVC1 and PL2. A few species of this genus are well known to perform denitrification [156-158] and they were also previously found in cathodic
biofilms performing nitrate reduction [93, 154]. A high abundance of OTUs belonging to the genus *Paracoccus* were also found at the PL1 and PL3 electrodes, although the role of denitrification on those reactors seem to be shared also by other groups of organisms such as *Dechloromonas* [159] (22 and 31% abundance respectively). Other species belonging to the family Rhodocyclaceae (other than *Dechloromonas* sp.) were previously isolated and/or enriched from Rice Paddy Soil [160-162], demonstrating also strong denitrifying activity.

*Stappia* sp. (present in all reactors, but especially encountered at 13 and 8% abundance in the reactors RVC2 and PL1 respectively), were also previously described to perform nitrate reduction [163, 164]. In addition, another genus identified in our reactors (*Truepera* sp.) was previously found in biological filter performing simultaneous nitrification and denitrification [165], and, more interestingly, also detected during cathodic denitrification[166] and performing nitrite reduction in BES[167].

Members of the Phylum Bacteroidetes (i.e. *Moheibacter* sp. and *Proteiniphilum* sp.) were also detected in relatively high abundances in all the reactors. Organisms of this Phylum are known for production of hydrogen during biomass fermentation [168]. A previous detailed assessment of a hydrogenase gene (by designing a specific probe), in conjunction with 16S rRNA gene analysis, revealed that actually only a small portion (12-33%) of OTUs recovered during hydrogen production were actually known strains, whereas a great number of organisms containing the hydrogenase gene was found to belong to unclassified genus from the phylum Bacteroidetes [168]. More specifically, species such as those belonging to the *Moheibacter* genus were previously isolated from sediments, and although only two species have been described to date, they were not reported as able to perform nitrate reduction [169, 170]. Therefore, since Bacteroidetes comprised from 3 to 7% abundance of organisms from all reactors, it is possible that they play a role on electron transfer via hydrogen production from the cathode in the present study.
Figure 20 – Most common genus identified in the reactors and its relative abundance within the biofilm microbial communities. Rhodocyclaceae* indicate genera other than Dechloromonas and Proteiniphilum.

As expected, methanogens were not encountered at significant abundances on plates or RVC electrodes (<2.6%). In fact, methanogenesis activity is long known to be inhibited during denitrification [171, 172]. More importantly than the presence of nitrate itself, denitrification transient products such as nitrite and nitric oxide seem to impose toxic effects to methanogens [108, 172]. Furthermore, the occurrence of autotrophic denitrification with hydrogen as electron donors can potentially decrease hydrogen partial pressure in the reactors to such a low threshold that can prevent hydrogenotrophic methanogenesis activity [171]. In fact, amongst RVC and plate reactors, PL2 showed the highest abundance of methanogens (2.6 % Methanobrevibacter), whereas all other reactors contained only less than 0.4% abundance of organisms of the same genus. This finding corroborates to additional results of greenhouse gases measurements, in which insignificant amounts of methane detected in most reactors), with an exception of S1. In reactor RVC1, the amount of measured methane (69.9 ±27.8 µmol) accounts for 12.9% ± 1.2 of electrons provided electrochemically, which helps explaining the low coulombic efficiency of this reactor. We hypothesise herein that the low coulombic efficiency in this
reactor (enabling free hydrogen within the biofilm) could still enable the hydrogenotrophic methanogenesis activity, but this effect should be further investigated, since the duplicate reactor RVC2 did not show significant amount of methane.
7 Demonstrating and optimising a lab scale upflow (membraneless) BES as a polishing mechanism for nitrate removal from real freshwater secondary (treated) effluents from WWTP – a freshwater study

This chapter addresses the Research Objective 3.1, aiming to investigate and demonstrate the feasibility of applying an easy-to-operate upflow cylinder BES as a polishing mechanism for treated secondary clarified effluent from a municipal WWTP, containing low levels of organic matter, low buffer capacity and low concentrations of remaining nitrate. The chapter demonstrates the feasibility of removing low levels of nitrate in the proposed process and includes a study on ion transfer mechanisms in its operation with freshwater (low conductivity) streams. A description of the materials, reactors design, and overall methodology used to assess this Research Objective were previously described in Chapter 4, and a detailed description of the specific operation procedures done to pursue this objective (including media composition and real wastewater description) are presented in Chapter 7.1. Following that, the research outcomes are then presented in Chapter 7.2, whereas additional perspectives for the technology and the main conclusions of the study are combined in Chapters 9 and 10 respectively.

7.1 Specific Experimental Setup

7.1.1 Reactor Inoculation and Operation

The cathode electrode was inoculated with biomass obtained from a previously operating denitrifying BES. The reactor was initially fed for 30 days (growth phase) at a flow rate of approximately 1 L d\(^{-1}\) with synthetic media containing 0.1 g L\(^{-1}\) MgSO\(_4\) \(\cdot\)7H\(_2\)O, 15 mg L\(^{-1}\) CaCl\(_2\) \(\cdot\)2H\(_2\)O, 1 g L\(^{-1}\) NaHCO\(_3\), 20 mg L\(^{-1}\) NO\(_3^–\)-N, 19.4 mg L\(^{-1}\) NaH\(_2\)PO\(_4\), 3.84 mg L\(^{-1}\) NH\(_4\)Cl, 19 mg L\(^{-1}\) KCl and trace elements solution as previously described [146]. After this period, the clarified secondary effluent from a WWTP started being used as feed.

The clarified effluent from WWTP was tested (n=4) for the presence of NO\(_3^–\)-N, NO\(_2^–\)-N, NH\(_4^+\)-N, Total Organic Carbon (TOC), Total Inorganic Carbon (TIC), Volatile Fatty Acids
(VFA) and Alcohols. The characterization indicated the presence of $2.5 \pm 0.5 \text{ mg L}^{-1} \text{NO}_3^-$ - N, $0.2 \pm 0.1 \text{ mg L}^{-1} \text{NH}_4^+$ - N, $36 \pm 5 \text{ mg L}^{-1} \text{TIC}$ and $9.0 \pm 0.7 \text{ mg L}^{-1} \text{TOC}$. Concentrations of VFA and Alcohols were below detection limit of the method at all times, confirming that only non-readily available organic matter was present in the clarified wastewater. Dissolved oxygen (DO) was also monitored in the inlet of the BES reactor and preliminary results indicated high oxygenation had occurred during wastewater collection in the treatment plant. Thus, the wastewater was sparged with Nitrogen gas until oxygen levels were below 1 mg L$^{-1}$ O$_2$. The reactor’s performance was evaluated through electrochemical measurements and by analysing liquid samples taken above (after) the cathodic zone.

**Mixing Conditions**

In order to investigate whether convection plays a role on ion transfer between cathode and anode, we checked whether current generation and potential losses across the liquid phase (within cathodic, anodic and gap areas) are affected by mixing conditions. A series of recirculating/feeding conditions were tested. Each mixing condition was set as a different combination of presence/absence of feed and recirculation circuits C1 and C2, where C1 comprises a recirculation circuit within both anode and gap areas and C2 comprises a recirculation circuit within cathodic area only (Figure 9). The tested mixing conditions in the freshwater study presented herein included: (Test 1, full mixing): feed + C1 + C2; (2) feed + C2; (3) feed only, no recirculation circuits operating; (4) no feed, no recirculation circuits operating. Each mixing condition was kept for 1 hour at a flow rate of 14.4 L d$^{-1}$, which allowed the cathodic media to be completely replenished (> 3 cathodic HRTs) before measuring the Ohmic potential losses across liquid phase – except for Test 4 which was carried out in the absence of feed.

### 7.2 Research Outcomes

#### 7.2.1 Denitrification rates from clarified secondary effluent in the BES reactor

Results presented herein include the BES reactor electrical response since the clarified effluent started to be used as feed (day zero). The feed flow rate was gradually increased
from 1.5 to 14.4 L d⁻¹, as indicated in Table 7. Each increase in flow rate was done after sampling the reactor until reaching the flow rate of 3.85 L d⁻¹ – phases 1 – 4. Since very fast flow rates were further tested in the end of the operation period, they were only tested for a short period of time due to the very short hydraulic retention times (HRTs) which led to rapid establishment of steady state conditions. Thus, a flow rate of 7.2 L d⁻¹ was kept for at least 1.5h, whereas a flow rate of 14.4 L d⁻¹ was kept for 1h, both corresponding to (at least) 3 HRTs. This procedure enabled complete media replenishment within the catholyte and guaranteed independence of results at each phase (5 – 8).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Flow rate (L d⁻¹)</th>
<th>Time range (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>0-4</td>
</tr>
<tr>
<td>2</td>
<td>2.16</td>
<td>4-12</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>12-18</td>
</tr>
<tr>
<td>4</td>
<td>3.85</td>
<td>18-19</td>
</tr>
<tr>
<td>5*</td>
<td>7.2</td>
<td>37.0 - 37.16</td>
</tr>
<tr>
<td>6*</td>
<td>7.2</td>
<td>37.16 - 37.2</td>
</tr>
<tr>
<td>7*</td>
<td>14.2</td>
<td>37.2 - 37.26</td>
</tr>
<tr>
<td>8*</td>
<td>14.2</td>
<td>37.26 - 37.32</td>
</tr>
</tbody>
</table>

Table 7 – Days of operation and applied flow rate before each sampling time. Asterisks indicate samples were taken at the same day, after running the reactor at the selected flow rate for at least 3 times the HRT.

Figure 21 highlights the results of experimental phases with increasing flow rates as previously mentioned. Current increased from phase 1 until phase 5 (4.0 to 5.7 mA respectively). Because of the increasing flow rate, the nitrogen loading rate also increased over time (Figure 21A) from 0.027 Kg N m⁻³ NCV d⁻¹ in the beginning of the experiments to 0.221 Kg N m⁻³ NCV d⁻¹ at phase 8. As shown in the graph, nitrogen removal rates (0.018 – 0.121 Kg N m⁻³ d⁻¹) were following the increasing nitrogen loading rates, indicating the biofilm was able to respond well to the increasing flow rates. Concentration of nitrogen compounds in the effluent decreased to nearly zero in the 19th day of operation at phase 4 (Figure 21B). However, from phase 5 onwards, the percentage of nitrate found in the
effluent – compared to that of the total influent nitrogen – started to increase again, indicating that the biofilm was reaching its maximum capacity at the tested conditions. Naturally, a further increase to 14 L d\(^{-1}\) led to a rather higher percentage of nitrate which was not removed.

Removal rates presented herein are within the ranges reported in previous BES studies [70, 75, 145], despite the lack of membrane (commonly used in BES to separate cathodic and anodic reactions), the absence of organic matter as electron donor and the low conductivity (< 2 mS cm\(^{-1}\)) of the wastewater feeding the system proposed herein. In fact, it has previously been demonstrated that cathodic denitrification is possible in low ionic strength streams, although removal rates can be affected by low conductivity and low buffer capacity [54].

Figure 21 – Reactor operation with different flow and N loading rates. (a) Current production (mA), Nitrogen loading rate and Nitrogen removal rate (Kg NO\(_3^-\) -N m\(^{-3}\) d\(^{-1}\)); (b) Mass balance (%) of nitrogen species leaving the reactor, to that of total nitrogen entering the system. NO\(_3^-\) -N, N\(_2\)O -N, NO\(_2^-\) -N and NH\(_4^+\) -N were measured from liquid samples, whereas N\(_2\) was assumed to be a result of complete denitrification happening in the system and calculated as all unaccounted nitrogen in liquid samples.
As indicated in Table 8, nitrate concentration of the wastewater entering the reactor was very low, as it was expected as a (treated) secondary clarified effluent. However, the BES system was able to further reduce the nitrate concentration to values lower than 1 mg L\(^{-1}\) or nearly zero at all times. Nitrite was present at very low concentrations in the inflow and not detected in the outflow of the reactor. Furthermore, analysis of greenhouse gases N\(_2\)O and CH\(_4\) also indicated negligible amounts of both compounds (<0.5% N\(_2\)O - N generated from influent nitrogen and <0.01 µM CH\(_4\) being generated at all times) (data not shown). Ammonium concentration tended to increase slightly in the beginning of the experiments (phase 1), indicating that dissimilatory nitrate reduction to ammonium was happening to a certain extent [120] and biofilm had not yet reached a steady state. However, interestingly, ammonium entering the reactor started being consumed from phase 2. Although it is possible that small concentrations of oxygen in the influent may be enabling sequential nitrification and denitrification [88, 173] to occur in different portions of the cathode, it is worth noting that ammonium concentration in the influent of phase 4 was already very small, as indicated in Table 8. The small concentration of nitrogen species entering the reactor in this phase – which is also demonstrated in the slightly decreased nitrogen loading rate (Figure 21A) – explains the very low concentration of dissolved nitrogen species that were detected in the effluent in phase 4.

Table 8 – Nitrogen species concentration (mg L\(^{-1}\) N) in the inflow, after passing through the cathodic zone of the BES reactor, and the concentration of removed/generated nitrogen in the reactor (where \(\Delta =\) outflow N − inflow N).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Inflow nitrogen species concentration (mg/L)</th>
<th>Outflow nitrogen species concentration (mg/L)</th>
<th>(\Delta) N (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO(_2) - N</td>
<td>NO(_3) - N</td>
<td>NH(_4) + N</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>2.8</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>2.1</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.0</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>0.0</td>
<td>1.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>
The pH of the clarified wastewater entering the reactor (6.9 ±0.1) tended to increase slightly after the cathodic zone due to denitrification/reduction reactions (Figure 22A). An important aspect to consider is the fact that the BES in the present study was operating with no additional buffer capacity to the amount already contained in the treated wastewater. However, the system was proven to work as a N-polishing mechanism, indicating that the buffer naturally contained in treated effluent was sufficient to neutralise the alkalinity generated by cathodic denitrification.

![Figure 22](image)

**Figure 22** – pH profile (A); Cell voltage and energy consumption (B) during nitrogen removal reactions in the upflow BES.

7.2.2 Potential losses and Pseudo-Ohmic resistances

Calculated total electrolyte’s Pseudo-Ohmic resistance ranged from 127 ±3 to 152 ±2 Ω, and varied between different regions of the reactor. Furthermore, current generation and electrolyte’s pseudo-Ohmic resistance within cathode and anode areas were greatly
affected by mixing conditions (Figure 23A and B). When applying both recirculation circuits 1 and 2 together with the feed in the first hour of experiment, the resistance was kept to a minimum of 43 ±1 and 34 ±1 Ω within anode and cathode respectively. The resistance then increased stepwise by sequentially interrupting recirculation within the different regions until reaching a maximum of 51.0 ±0.5 and 49.30 ±0.02 Ohms at anode and cathode respectively in the end of the test. The total resistance of the system was therefore decreased by 13.6 ±1.1% with the application of all recirculation circuits, compared to that of feed only. This decrease in pseudo-ohmic resistance in the system with added recirculation circuits indicates a non-Ohmic behaviour and confirms that convection plays an important role together with migration in the transport of ions within those regions. Thus, the improvement of reactor’s performance with added recirculation was likely due to better transport of ions to/from the bulk to the biofilm, confirming the importance of mixing/agitation to the operation of BES reactors with low conductivity.

However, although the resistance of the gap area was shown to be the highest of all regions, it seemed not to be affected by recirculation conditions. Furthermore, as gas is expected to be the end product of both denitrification (N₂) and anodic abiotic reactions (generating O₂), improvement on the transport of ions could be due to bubble removal, exposing again the electrode’s surface to the contact with the liquid phase.
Figure 23 – (Bio)electrochemical response at different recirculation regimes. (a) Current generation; (b) cathodic, anodic and gap pseudo-ohmic resistances. Recirculation: (1) Feed + circuit 1 + circuit 2; (2) Feed + circuit 2; (3) Feed only; (4) No feed, no recirculation.

Energy consumption based on the amount of nitrogen removed was only calculated for the experimental phases 1-8 presented in Section 7.2.1, and tended to decrease over time of operation (Figure 22B). Although no liquid samples were analysed during the short term recirculation tests, calculated energy consumption based on volume of treated effluent when applying the best operational conditions herein (full recirculation) indicated a total consumption of 0.027 ± 0.001 kWh m\(^{-3}\). However, 13.5 ±0.4 % of this volumetric energy consumption was in fact due to potential losses within cathode-anode gap (0.0037 ±0.0003 kWh m\(^{-3}\), corresponding to a total Cell Voltage loss of 0.334 ±0.013 V). Thus, as this region of the reactor is not playing a role on the reactions, the total energy spent for the treatment of the freshwater stream with low conductivity could be reduced by decreasing the distance between anode and cathode electrodes to a minimum. However, although oxygen concentrations of the effluent (after the anodic zone) were generally very low in the present study – and therefore would only have a minimum effect on cathodic
current generation or biofilm denitrification activity – care should be taken to avoid oxygen back diffusion from the anode to the cathode in case oxygen starts being generated in the counter electrode.
8 Demonstrating and optimising the proposed lab scale upflow BES prototype for nitrate removal from synthetic RAS – a seawater study

This chapter addresses Research Objective 3.2. As discussed in the literature review, maintaining low concentrations of nitrogen compounds (ammonium, nitrate or nitrite) in recirculating aquaculture waters is extremely important for a larger and healthier fish production, as well as for discharge purposes. Although ammonium removal from aquaculture streams is usually done within a nitrifying step, nitrate removal via denitrification is still partially limited by low organic matter availability. Thus, an easy-to-operate autotrophic denitrifying bioelectrochemical system is herein proposed for the treatment of seawater aquaculture streams. This chapter includes a study on ion transfer mechanisms in the proposed BES (in its operation with saltwater streams) as well as a comprehensive study on buffer capacity dependence of the BES and a description of the microbial community involved in this novel saltwater operation. A description of the materials, reactors design and overall methodology used to assess this Research Objective were previously described in Chapter 4, and a detailed description of the specific operation procedures done to pursue this objective (including media composition and real wastewater description) are presented in Chapter 8.1. Following that, the research outcomes are then presented in Chapter 7.2, whereas additional perspectives for the technology applicability and the main conclusions of the study are combined in Chapters 9 and 10 respectively.

8.1 Specific Experimental Setup

8.1.1 Synthetic saltwater medium

The synthetic aquaculture seawater used in the experiments was composed of 35 g L\(^{-1}\) sea salt (Ocean Nature Sea Salt, Aquasonic, Australia), 20 mg L\(^{-1}\) NO\(_3^-\) -N, 1 g L\(^{-1}\) NaHCO\(_3\), 19.4 mg L\(^{-1}\) NaH\(_2\)PO\(_4\), 3.84 mg L\(^{-1}\) NH\(_4\)Cl and trace elements solution as previously described [146]. The pH of the medium was adjusted to approximately 6.9 by adding HCl 1M, unless stated otherwise.
8.1.2 Reactor inoculation

The reactor was initially inoculated with 7 mL of a denitrifying inoculum coming from a fed-batch enrichment culture (as described in Section 4.4), containing approximately 2480 mg L\(^{-1}\) COD. As part of the inoculation procedure, the reactor was fed with the effluent from a previously running cathodic denitrifying parent reactor for approximately 2 months, and enriched in continuous mode using buffered synthetic media (described in Section 4.3.1) during preliminary operation, during which several preliminary experiments were carried out. After approximately one year, the reactor was shifted to saltwater operation by gradually increasing salt concentration to 35 g L\(^{-1}\) sea salt. During the adaptation, the reactor was then re-inoculated with sediments taken from an intertidal mangrove environment (5 - 40 cm deep) in Southeast Queensland, Australia. Sub-samples were taken throughout the whole depth of the collected core sample, mixed together and suspended in artificial brackish water containing 25 g L\(^{-1}\) sea salt (Ocean Nature Sea Salt, Aquasonic, Australia), 20 mg L\(^{-1}\) NO\(_3^-\)-N, 1 g L\(^{-1}\) NaHCO\(_3\), 19.4 mg L\(^{-1}\) NaH\(_2\)PO\(_4\), 3.84 mg L\(^{-1}\) NH\(_4\)Cl and trace elements solution as previously described \[146\]. The pH of the medium was adjusted to 6.9 by adding HCl 1M. The suspended material was then allowed to settle overnight at 3\(^\circ\)C for the removal of heavier particles. After that, the supernatant was collected and centrifuged at 12,000 rpm for 10 minutes. The sediment fraction of the centrifuged material was then re-suspended in 10 mL synthetic brackish water and inserted in the reactor through a port within the mid portion of the cathodic bed.

The synthetic seawater aquaculture medium used for the experiments was the same brackish water medium described above, except for a higher sea salt concentration of 35 g L\(^{-1}\). The medium was fed continuously from the bottom of the reactor, flowing first through the cathodic zone and then towards the anodic zone at an initial flow rate of 3 L d\(^{-1}\).

The following experimental sequence was developed to evaluate individually the effect of influent flow rate, added bicarbonate and of mixing conditions. The tests were done after 135 days of reactor operation.
8.1.3 Flow rate and Hydraulic Retention Time (HRT) variation

To determine the effects of different nitrate and buffer loading rates on current production and N-products profile, a series of feed flow rates and hence HRT were tested. Starting from the fastest feed rate (which indirectly enabled higher buffer loads to pass through the cathode), the flow rate was decreased on a daily basis (3, 2, 1 and 0.5 L d⁻¹, corresponding to cathodic HRT of approximately 40, 60, 120 and 240 min respectively), followed again by stepwise daily increase in flow rate to test the reproducibility of the results. During Test 1, recirculation was applied within the cathode electrode (recirculation circuit 2) only, to avoid mass transfer limitations. Liquid samples were taken twice at each condition (approximately 16 and 24 hours after setting up a new flow rate).

8.1.4 Electrolyte bicarbonate concentration

Based on previous work done on microbial anodes and cathodes [174], it is hypothesised that the electron transfer rate at the cathode is controlled not by the nitrate loading, but by the supply rate of pH buffers. Thus, the following set of tests was done in order to understand the effects of different buffer concentrations and assess whether higher buffer concentrations could improve current generation and denitrification rates. Since no buffer capacity other than that provided by bicarbonate system was available in the present study, the role of its presence to current generation and denitrification reactions were then evaluated by adding 1, 2, 4 and 6 g L⁻¹ sodium bicarbonate, corresponding to a final concentration of 14.3, 26.2, 50.0 and 73.8 mM (which includes the 2.3 mM bicarbonate present in the commercial sea salt added). Thus, the procedure theoretically increased the availability of influent carbonic acid and buffer capacity at a fixed flow rate of 3 L d⁻¹ and influent pH 7.1 ± 0.2. Recirculation within cathode electrode was kept at all times to avoid mass transfer limitations. Nitrate concentration in the feed was 20 mg L⁻¹ NO₃⁻ -N when the lowest concentrations of 1 and 2 g L⁻¹ sodium bicarbonate were used, whereas it was increased to 40 mg L⁻¹ NO₃⁻ -N when the highest concentrations of sodium bicarbonate were tested, to avoid nitrate limitation.
Effective buffer capacity considerations

As previously mentioned, the added sodium bicarbonate is expected to dissociate in the medium (Equation 14). In ideal (standard) conditions, the pKa₁ and pKa₂ values of the bicarbonate system (i.e. 6.35 and 10.2 respectively) dictate the relative amount of each dissociated form, depending on the actual pH of the solution. If the solution has neutral pH close to pKa₁, it is expected that approximately 50% of added sodium bicarbonate will dissociate into carbonic acid (CO₂/H₂CO₃) and 50% into bicarbonate (HCO₃⁻), while negligible CO₃²⁻ will be present. The effective buffer capacity is therefore considered to be the concentration of protons (mM H⁺) that can potentially be yielded during the conversion of H₂CO₃ to HCO₃⁻. At higher pH values that are closer to pKa₂ (as it may occur at exit of cathode zone due to denitrification activity), part of HCO₃⁻ can also lose protons and be further converted to CO₃²⁻, providing additional buffer capacity at higher pH.

However, in real conditions, the effective buffer capacity of bicarbonate system may be affected by the presence of other salts. As previously reviewed by Batstone et al [175], non-ideal behaviour of physicochemical processes such as ion pairing and speciation can significantly influence wastewater treatment (i.e. interfering on precipitation) [176] and should also be considered. Therefore, since the media used in this set of experiments contained high ionic strength characteristic of seawater (>0.6), bicarbonate speciation and medium effective buffer capacity was assessed by using the Minteq software (Visual MINTEQ 3.1, J.P. Gustafsson, Sweden), using the Debye-Hückel method for activity correction. As input parameters, the total concentration of most important ions in the medium (>0.05 mM) were considered, calculated as the sum of ions provided by both seasalt and added ions (according to described media composition). The input data of ions concentration in the prepared synthetic basic media (with added 1 g L⁻¹ sodium bicarbonate) are as described in Table 9.
Table 9 – Concentration of ions in the basic synthetic seawater media prepared for the assessment of the Research Objective 3.2.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mM)</th>
<th>Ion</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>544.2</td>
<td>Sr²⁺</td>
<td>0.09</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>28.2</td>
<td>Br⁻</td>
<td>0.3</td>
</tr>
<tr>
<td>Na⁺</td>
<td>480.2</td>
<td>PO₄³⁻</td>
<td>0.16</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>52.8</td>
<td>NH₄⁺</td>
<td>0.07</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>10.2</td>
<td>CO₃²⁻</td>
<td>14.3</td>
</tr>
<tr>
<td>K⁺</td>
<td>10.2</td>
<td>NO₃⁻</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Considering the measured inflow and outflow pH of the medium and the amount of bicarbonate added, proton availability was then modelled with the Minteq software. Moreover, considering 1 proton available per CO₂ in influent medium and 1 proton yielded per CO₃²⁻ formed in effluent medium, the theoretical generated current based on buffer capacity can be calculated as:

\[
I(\text{mA}) = \frac{Fl * (CO_{2\text{in}} + CO_{3\text{2-out}}) * e^- * F}{86400}
\]

where \(Fl\) is the influent flow rate (L d⁻¹), \(CO_{2\text{in}}\) (mM) is the concentration of CO₂/H₂CO₃ acid form which is present in the prepared media at the set pH (given by the Minteq simulation, considering the influent pH), \(CO_{3\text{2-out}}\) (mM) is the concentration of CO₃²⁻ basic form present at the exit from the cathode (given by the Minteq simulation, considering the effluent pH) and \(e^-\) is the amount of electrons (mM) required for the reduction of available protons (1 electron / proton).
8.1.5 Mixing conditions

In order to investigate whether convection plays a role on ion transfer between cathode and anode, we checked whether current generation and potential losses across the liquid phase (within cathodic, anodic and gap areas) are affected by mixing conditions. A series of recirculating/feeding conditions were tested. Each mixing condition was set as a different combination of presence/absence of feed (3L d⁻¹) and recirculation circuits C1 and C2, where C1 comprises a recirculation circuit within both anode and gap areas and C2 comprises a recirculation circuit within cathodic area only (Figure 9). The different tests included: (Phase 1) feed + C2; (Phase 2, full mixing) feed + C1 and C2; (Phase 3) feed + C1; (Phase 4) feed only; and (Phase 5) no feed and no recirculation, as summarised in Table 10. Each condition was kept for at least 3 hours, which allowed the cathodic media to be completely replenished (> 3 cathodic HRTs) before measuring the Ohmic potential losses across liquid phase – except for Phase 5 which was carried out in the absence of feed.

Table 10 – Summary of experimental mixing conditions applied during test 2.

<table>
<thead>
<tr>
<th>Test</th>
<th>Feed</th>
<th>Anode recirc. (circuit 1)</th>
<th>Cathode recirc. (circuit 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

8.2 Research Outcomes

8.2.1 Tests at different influent flow rates

The results of Test 1 obtained at different flow rates and HRTs are shown in Figure 24. Liquid samples during this test were taken twice at each flow rate, and in each direction of the test (increasing/decreasing flow rate). Therefore, standard deviations presented herein are calculated for the four samples, unless stated otherwise.
Current reached a maximum of 7.2 mA in the beginning of the first run at 3 L d⁻¹, then decreased to a minimum of 3.9 mA at the flow rate of 0.5 L d⁻¹. A further decrease in current was observed when the flow rate was 1 L d⁻¹, which happened likely due to air intrusion in the system when replacing the feed reservoir.

Nitrate concentration in the reactor’s effluent was 12.2 ± 0.9 mg L⁻¹ NO₃⁻-N at the fastest flow rate, whereas only 2.6 ± 1.3 mg L⁻¹ NO₃⁻-N was leaving the reactor at the lower flow rates. A higher percentage of intermediate nitrite was also detected in the effluent at fast flow rates, especially in the first run of the experiment (3.3 ± 0.4 mg L⁻¹ NO₂⁻-N at 3 L d⁻¹, n=2), however a relatively lower concentration (0.4 ± 0.2 mg L⁻¹, n=2) was present at the same flow rate at the end of the experiment. As previously shown, nitrate is a preferable electron acceptor during denitrification partially due to its higher redox potential compared to that of nitrite (E₀ NO₃⁻ = + 0.43 V vs SHE; E₀ NO₂⁻ = + 0.35 V vs SHE) and nitrite depletion is more likely to happen after depletion of nitrate, as previously observed [95, 96]. Noteworthy, nitrite imposes acute toxicity to fish and its presence in fish tanks (from either nitrification or denitrification intermediates) can be detrimental for the production [43]. However, our results show that, if operated at the optimal nitrate loading rate, the proposed BES is also able to remove nitrite (as previously demonstrated by Virdis et al [53]), which can be a convenient feature for removal of nitrite left over from the aquaculture nitrifying step.

On the contrary, the highest levels of nitrous oxide (3.2 ± 0.15 mg L⁻¹ N₂O-N, corresponding to 16.7 ± 0.6 % of influent nitrogen added as nitrate) occurred at the flow rate of 0.5 L d⁻¹ instead, as opposed to only 6.6 ± 2.4 % of added NO₃⁻-N at 3 L d⁻¹. Nitrous oxide is often observed in BESs and its accumulation is generally related to a mismatch between its consumption rate and that of its generation during the denitrification process [53, 96]. Thus, the highest accumulation of N₂O at slow flow rate in the present study can be related to the larger amount of nitrate and, particularly that of nitrite been reduced during this experimental condition, shifting the denitrification process towards the nitrous oxide formation step, which was not stoichiometrically balanced to its reduction to di-nitrogen gas. Importantly, nitrous oxide is observed to accumulate at all conditions tested, which signifies that its reduction rate was slower than that of all the other
denitrification steps at all times. These results indicate that the best efficiency in removing nitrogen in the system was achieved at the lowest flow rate (65 ± 7 % nitrogen removal at 0.5 L d⁻¹). Furthermore, this relatively low nitrogen removal efficiency was a result of intermediate compounds accumulation, especially nitrous oxide.

Although high rates of ammonium formation had been previously reported in Chapter 5, this process did not occur at significant rates in the present study. Overall, only 0.45 ± 0.2 mg L⁻¹ NH₄⁺ -N was detected during the experiments, which represents only 2.4 ± 1.1 % of nitrate added being converted to ammonium at all times. As previously demonstrated, high ratios of electron donors (i.e. H₂) to that of electron acceptors (i.e. NO₃⁻ -N) available during biological nitrate reduction can stimulate ammonium generation via Dissimilatory Nitrate Reduction to Ammonium. Thus, cathodic hydrogen formation – and consequently ammonium formation – were minimised herein by enabling a preliminary biofilm growth/adaptation (and thus the electron uptake capacity of denitrifiers) and by increasing the applied cathodic potential from -0.9V vs SHE in the previous study to -0.7 V vs SHE in the present study. Therefore, only a maximum of 0.85 mg L⁻¹ NH₄⁺ -N was formed in this study.

Nitrogen removal rate via complete denitrification was at its maximum at 2 and 3 L d⁻¹ (0.13 ± 0.01 kg N m⁻³ NCV d⁻¹, corresponding to 0.01 kg N m⁻³ TVC d⁻¹, with no significant difference between these two flow rates) and decreased to 0.07 ± 0.01 kg N m⁻³ d⁻¹ at 0.5 L d⁻¹. Those nitrogen removal rates – per net cathodic volume (NCV) – are within the ranges previously reported in the literature for bioelectrochemical denitrification [53, 70, 76], which proves the proposed upflow membraneless setup as a viable system for the removal of nitrate from saltwater streams.

Although better removal rates were observed at 3 L d⁻¹ (0.7 kg N m⁻³ d⁻¹ loading rate), only less than 20% of NO₃⁻ -N was removed, indicating that nitrate loading rates of 0.11 and 0.23 kg N m⁻³ d⁻¹ are best indicated for this purpose, as only minimum amounts of nitrite accumulated and best nitrate removal efficiencies were achieved. The accumulation of nitrate and nitrite at the fast flow rate indicate that nitrate loading rate was not the rate limiting of the process.
Minteq simulations indicated that approximately 10-18% of added sodium bicarbonate is expected in acid form (CO$_2$) and these values were strongly affected even by small variations within influent pH. After passing through cathodic zone, pH increased immediately (Figure 24C), which was inversely associated to flow rate, reaching 8.0 ± 0.1 at the lowest flow rate of 0.5 L d$^{-1}$. However, only a maximum of 1% of the bicarbonate system was predicted as CO$_3^{2-}$ by the software at the measured effluent pH, indicating that only a negligible buffer capacity was provided by protons coming from HCO$_3^-$ to form CO$_3^{2-}$. Interestingly, calculated current generation was lower than that obtained experimentally, but followed a similar trend at all times (Figure 24A), indicating that the effective buffer capacity is strongly linked to the system’s performance. These results also indicate a depletion of buffer capacity (proton availability from bicarbonate system), after which, the consumption of protons coming from water split rather than from CO$_2$ (which led to pH rise, as indicated in Figure 24C) could have allowed a higher current generation than originally predicted. After the anodic zone, pH tended again towards neutral values due to oxidation reactions at the anode (7.2 ± 0.3).

Measured anodic potential showed a positive correlation with Cl$_2$ concentration (Figure 24E). However, measured potentials were relatively constant at all times (+1.18 ± 0.02 V vs SHE) and lower than the standard redox potential for Cl$_2$ formation ($E^0 = +1.36$ V vs SHE)[67], which explains the very low concentration of Cl$_2$ detected in the effluent (0.07 ± 0.06 mg L$^{-1}$ Cl$_2$, which was very close to the lower detection limit of the method, despite the very high concentration of sea salt of prepared artificial media). Thus, although the possible Cl$_2$ toxicity (both to the BES system and the aquaculture fish tank) cannot be yet discarded at this stage, it is hypothesised that it would only play a minor role due to the very low concentrations detected. Nevertheless, Chlorine toxicity should be further assessed in pilot studies when integrating the BES within the aquaculture system.

Although measured DO was also very low (1.3 ± 0.7 mg L$^{-1}$ O$_2$), the cited anode potentials are higher than the standard potential for O$_2$ formation ($E^0 = +0.82$ V vs SHE) [1]. It is possible that bubble formation may have reduced the transfer rates of oxygen between electrode (at formation points) and liquid phase, which corroborates with a slightly larger dissolved oxygen concentration at smaller flow rates: longer HRTs would enable higher
transfer of oxygen from bubbles (formed at anode) towards liquid phase. Therefore, improvement of electrode configuration and mixing regimes enabling better transfer of oxygen to liquid phase are encouraged.

Overall energy consumption for nitrogen removal via denitrification (27 ± 6 kWh kg N\textsuperscript{-1}) varied according to cell voltage, especially when fast flow rates were applied, as indicated in Figure 24D. Although lowest cell voltages were obtained at the lowest flow rates of 0.5 L d\textsuperscript{-1}, a lower nitrogen removal per operation time was observed, which led to an increase in specific energy consumption at this flow rate.
Figure 24 – Reactor behaviour during Test 1, carried out with the flow rates of 3, 2, 1 and 0.5 L d\(^{-1}\), corresponding to 40, 60, 120 and 240 minutes HRT respectively. (a) Experimental and calculated current production based on maximum buffer capacity (mA), Nitrate removal rate via complete denitrification (kg NO\(_3\)-N m\(^{-3}\)d\(^{-1}\)) and nitrogen removal efficiency (%); (b) Nitrogen balance (%) of all nitrate entering the system. NO\(_3\)-N, N\(_2\)O -N, NO\(_2\) -N and NH\(_4\)+ -N were measured from liquid samples, whereas N\(_2\) was assumed to be a result of complete denitrification and calculated as all unaccounted nitrogen in liquid samples. (c) reactor pH profile at different sampling points: influent media, post-cathode media and post-anode media leaving the system; (d) Cell voltage (V) and calculated Energy Consumption (kWh kg NO\(_3\)-N\(^{-1}\)); (e) anodic reactions: anodic potential (V), Dissolved Oxygen (mg L\(^{-1}\) O\(_2\)) and free Chlorine (mg L\(^{-1}\) Cl\(_2\)).
8.2.2 Electrolyte buffer capacity

Although pH and buffer capacity of the synthetic media normally tested in BES systems are well known to influence the reactors performance [131, 177-179], the variability of media composition on the actual buffer capacity has not been evaluated so far. Therefore, this section provides detailed assessment of the effective buffer capacity and its effects on current generation and nitrate reduction rate when varying the electrolyte’s sodium bicarbonate concentration.

When applying only 1 g L\(^{-1}\) NaHCO\(_3\), the pH of inflow media increased from 6.9 ±0.0 to 8.0 ±0.0 right after the cathode. However, the pH increased from only 7.3 ±0.1 to 7.5 ±0.1 when 6 g L\(^{-1}\) NaHCO\(_3\) was tested (Figure 25). Based on pH measurements shown in Figure 25A and known concentration of major ions in prepared media, proton availability (mM H\(^+\)) and expected current production were calculated as shown in Figure 25B and C respectively. Although buffer capacity increases with addition of bicarbonate, Minteq simulations indicated that bicarbonate speciation and effective concentration of proton equivalents in the saltwater medium were strongly dependant on pH. As indicated, the added bicarbonate was increased by a factor of 6, whereas the effective buffer capacity was only doubled due to the slightly higher feed pH.

Effective current generation was in fact slightly higher than predicted, which reflects bigger pH changes between inflow and outflow at small additions of sodium bicarbonate, corroborating with tests at different flow rates. However, predicted and effective current values tended to match more closely at higher bicarbonate additions, and a minimum pH change was observed when adding 6 g L\(^{-1}\) sodium bicarbonate.

The increase in sodium bicarbonate concentration (from 1 to 6 g L\(^{-1}\) NaHCO\(_3\)) also led to an increase of denitrification rates from 0.14 ±0.01 kg N m\(^{-3}\) d\(^{-1}\) to 0.18 ±0.02 Kg N m\(^{-3}\) d\(^{-1}\) respectively (Figure 25C). However, the effective denitrification rates were smaller than expected at the maximum added buffer capacity, as a result of bigger nitrite accumulation (Figure 25D). Since nitrate is a more preferrable electron acceptor than nitrite, the higher...
nitrate loading rate likely stimulated its consumption thus causing nitrite accumulation. In addition, a slightly smaller coulombic efficiency occurred when adding 6 g L\(^{-1}\) sodium bicarbonate (90.8 ±1.5%, compared to 105.1 ±0.2 at 1 g L\(^{-1}\) sodium bicarbonate addition), indicating that more electrons were lost and not used for denitrification at high additions of bicarbonate.

Figure 25 – Effects of different buffer capacity concentrations. Tests were carried out with inflow concentrations of 1, 2, 4 and 6 mg L\(^{-1}\) NaHCO\(_3\). (a) pH profiles of the influent and cathodic effluent medium; (b) Experimental and calculated current production based on buffer capacity (mA), and maximum proton availability from bicarbonate buffer system at the influent and effluent pH; (c) Effective and expected denitrification rates and nitrate loading rate (Kg NO\(_3\)-N m\(^{-3}\)d\(^{-1}\)) and (d) Concentration of nitrate reduction products leaving the cathodic zone. The sum of all products represent the total nitrate reduced in the system.
Although previous studies clearly indicated the buffer capacity dependence on current generation in bioelectrochemical systems, to the best of our knowledge this is the first study demonstrating the direct relationship between effective buffer capacity (as H⁺ equivalents), current generation and maximum achievable denitrification rates. These findings may represent an important tool for the prediction of nitrate removal rates/efficiencies from wastewaters containing different salts concentrations and buffer capacities. In addition, this data is very useful in calculating the ideal nitrate loading rate, in order to maximize removal rates and avoid intermediates accumulation. However, since the method is based simply on influent medium characteristics, it assumes microbial activity is not a limiting factor. Thus, achieving maximum removal rates will in practice depend on a preliminary biofilm growth/adaptation. Moreover, as clearly indicated in the experiments with variable flow rate, the effective current generated can be slightly higher than predicted, happening at the cost of pH rise (Figure 24A).

8.2.3 Effects of mixing conditions

The current response to various mixing conditions within the reactor is presented in Figure 26A. During the initial phase of the experiment (Phase 1), only the feed (at 3 L d⁻¹) and the cathodic recirculation were applied and average current was 7.2 ±0.1 mA at time of sampling. By starting the anodic recirculation in Phase 2, the current increased to 8.6 ± 0.2 mA. The absence of cathodic recirculation was tested in Phase 3, in which the current decreased to 6.2 ± 0.2 mA compared to the previous condition. During Phase 4, both recirculation circuits 1 and 2 were absent and the current further decreased to 5.5 ± 0.2 mA. Furthermore, current was greatly affected in Phase 5 by the absence of both feed and recirculation and decreased to only 3 ± 0.1 mA. However, the experiment in Phase 5 was interrupted before reaching steady state to avoid potentially detrimental disturbance to the biofilm due to absence of feed, which could have led to a further decrease in current otherwise.

During the tests with different mixing conditions, cathode area posed the highest pseudo-Ohmic resistance of the system (5.9 ± 0.4 Ω), despite its depth being only 5 cm as opposed to 7 cm depth for both gap (3.8 ± 0.2 Ω) and anode bed (1.8 ± 1.5 Ω), Figure
23B. Although cathodic and gap resistance were very constant throughout the experimental conditions, anodic resistance was greatly reduced when starting anodic recirculation (Phase 2). Despite anodic recirculation operation also in Phase 3, anodic resistance increased slightly but further greatly increased in the absence of anodic recirculation and feed (Phases 4 and 5 respectively).

The flux of ions between the electrodes in electrochemical systems is driven by diffusion (activity gradient), migration (electrostatic potential gradient dependent on electrolyte resistivity) and convection (fluid flow) [128]. However, considering that (1) ions concentrations in bulk solution are high enough and (2) only minimal concentration gradients are happening at the diffusion layer (interface liquid x biofilm), then the flux of ions through diffusion in BES can be considered negligible. Moreover, the relatively constant cathodic and gap resistance with different mixing conditions in our study indicate the true Ohmic nature of the charge transfer, where ion fluxes are happening mainly through migration processes. Noteworthy, migration of ions is directly related to ionic conductivity (and inversely related to Ohmic resistance). Thus, considering that a seawater medium containing 35 g L⁻¹ sea salts present conductivity which is high enough to enable an efficient migration ion transport, it is understandable that no further reduction of electrolyte resistance will occur by applying the recirculation circuits. Therefore, although the recirculation circuit 2 around the cathodic area clearly improves current generation, the constant Ohmic resistance indicates that convection did not play a role in ion transport within the cathodic area and increased performance is attributed to improved mass transfer or flow distribution within the cathode rather than convective charge flow.
Interestingly (and differently from the freshwater operation presented in Chapter 7), minimum voltage loss was occurring at the gap area between cathode and anode, which accounted for only less than 1% of energy consumption in the saltwater study. This in fact indicates that an increase of a gap distance between cathode and anode may be feasible in saltwater denitrifying BES – if necessary to avoid oxygen return from the anode in the upflow membraneless configuration – without significantly affecting the system performance or the energy consumption.

Contrarily to a purely migration-driven process, a decrease in pseudo-Ohmic resistance at the anodic region was observed when recirculation circuit 1 was introduced within the anodic and the gap areas, indicating the presence of non-Ohmic behaviour. The improvement in current generation with recirculation circuit 1 is attributed to the removal of gas bubbles formed at the anode (i.e. Oxygen), which were preventing contact between electrode surface and the liquid phase, thus inactivating part of the anode electrode. Those results confirm the importance of convection processes through anodic recirculation to the system, which facilitates mass transport and gas dispersion within the anode. This
behaviour did not occur at the cathode electrode likely due to the cathode setup in which the granules were occupying the whole reactor’s cross-section, forcing the water to flow through (i.e. avoiding preferential flow) and likely removing the N₂ bubbles. Modelling this system with a Computational Fluid Dynamics (CFD) analysis could possibly assist in better understanding the extent of migration and diffusion processes in the upflow configuration.

8.2.4 Microbial community composition

Analysis of the microbial community structure via sequencing of the 16S rRNA gene are summarised in Figure 5, which indicates that the most abundant taxa recovered from the cathodic denitrifying biofilm included members of the family Alteromonadaceae (21.4% of total Operational Taxonomic Units, OTUs), where 21% had 98.4 – 100% similarity to members of the genus *Marinobacter*. In addition, 17.5% of the OTUs belong to the family Rhodocyclaceae, whereas members of the families Flavobacteriaceae, Hyphomicrobiaceae and Marinicellaceae (unclassified family) comprised 8%, 4.8% and 6.6% of all recovered OTUs, respectively. The genera *Maricauda* sp. (7% relative abundance, with 98.4-100% similarity), and *Marinicella* sp. (6.5% abundance, 63-100% similarity) comprised the most abundant within the families Flavobacteriaceae and Marinicellaceae, respectively. The biofilm sample also indicated great variability of taxa, with majority of detected OTUs (37%) belonging to different families each of which accounted for only less than 1% of the organisms in the sample (Figure 27).

The most abundant genera detected in the cathodic denitrifying biofilm in the present work (*Marinobacter* sp., *Maricauda* sp. and *Marinicella* sp.) are known halotolerants and have been previously reported to grow in coastal sediments or deep seas and, especially species from *Marinobacter* genus, are known for its ubiquitous distribution in marine environments such as oceans, shallow seawaters, sand and hypersaline lakes [180, 181].

Although some organisms of the genus *Marinobacter* (family Alteromonadaceae) are typically known to be heterotrophs [180], many species such as *M. subterrani* and *M. aquaeolei* have been reported able to perform Fe (II) oxidation [182, 183]. In addition, *Marinobacter* sp. isolates previously reported in brackish water aquaculture ponds, were also suggested as being able to reduce nitrate to nitrite both in oxic and anoxic conditions.
Species such as *Marinobacter hydrocarbonoclasticus* are well known denitrifiers [180] and have been previously enriched in denitrifying reactors using Hydrogen as electron donors [185]. Similarly, some strains of *Maricaulis* sp. (family Hyphomonadaceae) are also known to reduce nitrate [186], whereas several species of the family Hyphomicrobiaceae (4.8% abundance) are known to perform nitrate reduction. More specifically, some species of *Hyphomicrobium* genus are also known to be able to use hydrogen as electron donor and/or perform complete denitrification [187]. Therefore, microorganisms belonging to the *Marinobacter* genus and to the families Hyphomicrobiaceae and Hyphomonadaceae are likely the key autotrophic denitrifiers in the present study.

![Microbial community structure](image)

Figure 27 – Microbial community structure (main Families and Genus, and their relative abundance) detected in the cathodic denitrifying biofilm treating synthetic seawater aquaculture stream.

A few *Marinicella* sp. (an unclassified genus belonging to the class Gammaproteobacteria) have firstly been reported as an aerobic bacteria unable to reduce nitrate [188]. However, organisms of this genus were previously detected with approximately 10% abundance within anaerobic reactors performing sulfide oxidation in the presence of nitrate as electron acceptor [163]. Similarly, although a recent study reported some members of *Maricauda* genus (Flavobacteriaceae) as unable to perform denitrification [189], a few species from this family (i.e. *Maribacter* sp.) were previously reported as nitrate reducers [190], or able
to perform complete denitrification (i.e. *Flavobacterium banpakuense*) [191]. Furthermore, other species from this family have been previously detected in engineered experimental soil columns performing denitrification [192], as well as in natural seawater environments [193, 194]. Moreover, to the best of our knowledge, only a few studies on the physiology of *Marinicella* and *Maricauda* species are available in the literature to date.

*M. hydrocarbonoclasticus* (specie closely related to the *Marinobacter* OTUs recovered in the present work) is known to possess the enzyme nitrous oxide reductase (N$_2$OR) [195], indicating they are likely playing a role on N$_2$O reduction in our system. Several genera within the families Rhodobacteraceae and Rhodocyclaceae (2.1% and 17% relative abundance, respectively) are known to reduce nitrate to nitrite [187]. However, only a few genera belonging to those families were reported to proceed complete denitrification, including *Paracoccus* sp. (Rhodobacteraceae) and *Thauera* sp. (Rhodocyclaceae) [156, 187], though these were present only in very small abundance within the cathodic biofilm. Although the incapacity of some organisms in reducing nitrous oxide may help understanding the imbalance between its formation and consumption within the biofilm, it is unclear from the available literature what is the role of remain recovered bacterial taxa in regards the reduction of nitrous oxide.

Although a few genera of the families Rhodobacteraceae (Alphaproteobacteria) [74, 154] and Rhodocyclaceae (Betaproteobacteria) [71, 74, 87, 93] have been previously identified in cathodic biofilms of bioelectrochemical systems, the community structure observed in our cathodic denitrifying biofilm differs considerably from that of other studies, which is attributed mainly to the different inoculum source and the high salinity of media used in the experiments herein.
9 Future perspectives of the upflow configuration

9.1 Freshwater: secondary effluent application

The upflow reactor showed an energy consumption of 23 kWh Kg N\(^{-1}\) at the optimum feed rate of 7.2 L d\(^{-1}\) (Phases 5 and 6 of the referred study presented in Chapter 7, which led to best nitrogen removal efficiencies with minimum accumulation of intermediates). This energy consumption is considerably higher than that required for complete nitrogen removal in activated sludge itself (ranging from 2.3 to 6.5 kWh Kg N\(^{-1}\)) [196]. However, as discussed in Section 2.1.1, an increase in the added organic matter such as methanol, and/or a substantial increase of the recirculation rates may be required to remove nitrate to levels lower than 1 mg L\(^{-1}\) NO\(_3^-\)-N in activated sludge plants, which may further lead to considerable increase in energy costs due to pumping and aeration of larger volumes of water.

Thus, since the removal of such small nitrogen concentrations is not recommended within the activated sludge itself, the main competing technology to the upflow bioelectrochemical system introduced in this work is the use sand(bio)filters as a polishing mechanism to further remove the low concentrations of nitrate leaving the main denitrification step in activated sludge WWTPs. Neglecting energy consumption for operation of pumps of both sand filters and BES, the operating costs of the two systems could be compared by assessing the costs of methanol addition for heterotrophic denitrification (sand filter) and the costs of electricity required for autotrophic denitrification (BES). Considering the overall market price of methanol as being approximately AUD$0.494 per kg methanol (according to Methanex Asia-Pacific prices for 2016 and the current exchange rates), and considering the stoichiometry of heterotrophic denitrification (Equation 4) in which a minimum of 2 kg methanol are required for the reduction of 1 kg NO\(_3^-\)-N, then the calculated price for methanol addition is approximately AUD$0.99 per kg NO\(_3^-\)-N removed. However, in practice, it is known that higher methanol addition is actually required (i.e. partly due to bacterial growth requirements or due to losses coming from oxygen consumption), as specified in the Section 2.1.1. Therefore, when considering that a ratio of at least 4/1 (kg methanol/ kg N) is required [17], then the actual cost would be doubled to approximately
AUD$2.00 per Kg NO₃⁻-N removed. Comparatively, when considering the energy requirements of the proposed upflow BES – including a 10% electricity loss due to alternate current (AC) to direct current (DC) conversion – and an overall (conservative) market price of electricity in Australia as being approximately AUD$0.15 per kWh, then the operational cost for nitrogen removal via autotrophic BES denitrification presented herein is AUD$3.45 Kg N⁻¹. Off-peak electricity price for industrial use can be significantly lower than this, possibly making the BES an attractive alternative to sand filters from an operating cost perspective.

Despite the competitive operational costs of the herein presented freshwater denitrifying BES, the Ohmic losses impose another challenge towards the application (and scale up) of the technology. Due to the low conductivity, the migration of ions occurring in the electrolyte between cathode and anode electrodes may be limited, imposing Ohmic losses that can affect not only the energy consumption, but also the denitrification rates, as the cathode is expected to show a profile of electrode potentials in the flow direction. As described in Section 4.6.2, by measuring the potential difference vertically between the top and the bottom of the 5 cm deep cathode (in the same direction of liquid flow), at a generated current of only 6.4 mA, an electrolyte Ohmic drop of 0.22 V was recorded despite the use of full recirculation regime during time of Ohmic drop measurements¹. As previously mentioned by Rozendal et al [117], the electrolyte Ohmic loss varies according to the distance (in the direction of ion flow), the current density and the electrolyte conductivity. Considering (1) a constant inflow conductivity and (2) a linear increase in current by increasing the cathodic depth (i.e. surface area), then a four fold Ohmic drop could be expected if the cathode depth was to be increased by only two times (to 10 cm). Thus, if an Ohmic drop of 0.22 V was originally measured for the 5 cm cathode bed, it

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¹ Electrolyte Ohmic drop of different areas of the upflow reactor and different mixing conditions were measured as part of Research Objective 3.1, and used for calculations of Pseudo-Ohmic resistances as shown in the results section of Chapter 7 (Item 7.2.2). The cathodic electrolyte Ohmic drop considered here corresponds to a full recirculation regime.
would then rise to approximately 0.88V. Although a potential of -0.9 V vs SHE was being applied at the cathode (controlled with the RE placed just above the cathode bed), this Ohmic drop would lead to an increase of the effective potential (at the bottom of the cathodic bed) to positive values up to 0 V vs SHE. However, cathodic denitrification activity was already previously shown to be severely reduced at this potential [86], indicating that the lower portion of the cathode would likely be inactive. Thus, increasing the cathode electrode surface area by adding more graphite granules to depths that are bigger than 10 cm will likely lead to no further improvement of performance. Moreover, whenever a higher current is generated (e.g. due to the use of better electrode materials that enable better electron transfer rates to the biofilm), an even bigger Ohmic drop can be expected due to the increased generation and transport of ions, possibly leading to the inactivation of even larger portions of the lower cathode (at positive potentials outside the required range for denitrification). Therefore, it becomes clear that a deficient performance can be expected when scaling up such systems in the vertical direction and it is recommended herein that scaling up should be done only in the cross-section (i.e. increasing the projected surface area without increasing the cathode depth).

9.2 **Saltwater: aquaculture application**

It is important to note that the saltwater and freshwater studies presented in this thesis were done in two different reactors (with different dimensions) and operated with different experimental conditions at the time of characterisation (i.e. different flow rates). In addition, achieved current was also different in the two systems, as well as the formation/accumulation of intermediate nitrogen compounds, whose formation does consume electrons (i.e. increasing current generation and energy consumption), although they were not removed from the system. Thus, the discussion of operational costs presented herein does not aim to provide a comparison between the saltwater and freshwater applications, but to present an overall guidance about the achieved performance of the upflow BES setup, providing means of comparison with other denitrification technologies and its integration in the aquaculture application.
Therefore, although the cell voltage of the saltwater operated reactor was lower than in the freshwater-operated reactor ($E_{cell} = 1.88$ V, as shown in Section 8.2.1 compared to $E_{cell} = 2.3$ V, as shown in Section 7.2.1, respectively), the overall energy consumption of the saltwater study at the optimum flow rate of 0.5 L d$^{-1}$ was approximately 28.8 kWh kg N$^{-1}$, which is in fact higher than that presented for the freshwater study. Noteworthy, as previously exposed in Section 7.2.2, a positive effect of mixing and feed flow rate in increasing ion transfer was observed during the operation of the system with low conductivity electrolyte. Thus, since a much faster flow rate was used at the time of energy consumption assessment in the freshwater study (7.2 L d$^{-1}$, compared to 0.5 L d$^{-1}$ in the saltwater study), it has certainly contributed to an increased ion transfer and reduced energy consumption in the freshwater study – which would likely had been much higher (with an even higher cell voltage) if a similar flow rate had been used. In addition, approximately 20% of influent nitrogen has accumulated as N$_2$O in the saltwater study, contributing to the higher energy consumption observed.

Considering the conservative 10% electricity loss due AC/DC conversion and the above mentioned electricity price for industries in Australia (AUD\$0.15 per kWh), the energy consumption for the saltwater operation represent an average cost of approximately AUD\$4.75 per kg N removed, which is currently more than twice as much as the calculated operational cost for the operation of sand filters (methanol addition for heterotrophic denitrification, AUD\$2.00 per Kg NO$_3^-$-N removed).

It is worth noting that standard costs for aeration of aquaculture fish tanks could be at least partially offset by taking into account the production of oxygen at the counter electrode (anode). Based purely on calculations which take into account the generated current and the Faraday’s law, oxygen concentrations could achieve levels – at the exit of the bioelectrochemical denitrification reactor – ranging from 16.4 mg O$_2$ L$^{-1}$ at 3 L d$^{-1}$ to concentrations above the oxygen saturation at 1 atm. Since producing oxygen above the saturation concentration is in practice not feasible, one could suggest a recirculation around the anode to include a circuit straight from the fish tank, thus diluting the generated oxygen in the water column and avoiding losses to the air phase. Although further studies are necessary for the improvement of oxygen diffusion from the anode to the water column.
(especially at such high oxygen production rate), the introduction of the electrochemically generated oxygen into the recycled stream could thus potentially at least partly reduce the need for further oxygenation of the recycled water, whose cost was previously estimated to be approximately 2.6 kWh kg O\textsubscript{2}^{-1} [197].

Although, as previously described in Section 9.1, cathodic beds with depths bigger than 10 cm are not recommended for freshwater operation due to the significative Ohmic drop, the operation of the reactor with saltwater stream at a set cathodic potential of -0.9 V vs. SHE led to only 0.05V Ohmic drop for the 5 cm deep cathode\textsuperscript{2} at a generated current of approximately 8.6 mA. Considering that the effective potential at the bottom of the cathode should not reach values more positive than 0 V vs SHE, a maximum depth of 20 cm could be applied for cathodic bed operated with saltwater streams without significantly losing denitrification activity. Based on the above considerations, a suggestion could be made for capital savings in these systems when used for the treatment of saltwater streams, namely the possibility of implementing the BES as an in-line in-pipe system where both cathode and anode are embedded in a pipe for capital cost savings. However, as previously mentioned for the freshwater application above, if higher currents are generated (as it would be desired), a bigger Ohmic drop is certainly also expected, leading to the inactivation of even larger portions of the lower cathode. Since a detailed assessment of MEC economic feasibility has been previously done [198, 199] and included both capital and operational costs, this aspect is only discussed shortly herein. The Ohmic resistance as calculated in the best proposed operational condition of the upflow reactor (full recirculation) enabled calculation of the total internal resistance of the saltwater system (33.3 mΩ m\textsuperscript{2}), which is in fact within the range previously suggested as economically attractive for microbial electrochemical systems [198]. Sleutels et al. also concluded that the current density of MEC needs to reach at least 20 A m\textsuperscript{2} in order to make the BES

\textsuperscript{2} Electrolyte Ohmic drop of different areas of the upflow reactor and different mixing conditions were measured as part of Research Objective 3.2, and used for calculations of Pseudo-Ohmic resistances as shown in the results section of Chapter 8 (Section 8.2.3). The cathodic electrolyte Ohmic drop considered here corresponds to a full recirculation regime.
technology economically attractive [198], which indicates that the current generation in our system (both for freshwater and saltwater denitrification) still need to be increased by at least 10 times in order to make this technology commercially attractive. However, since the referred study included an added value for hydrogen recovery at the cathode, it is likely that this required current density would be higher in the BES proposed herein – which does not focus on the recovery of valuable products. A comparison of the proposed upflow BES technology with other previously studied denitrification technologies can be found in Table 11.
Table 11 – Comparison between some previously studied competing denitrification technologies that could potentially be applied as polishing mechanisms for the removal of low levels of nitrate.

<table>
<thead>
<tr>
<th>Technology</th>
<th>DEN Rate Kg N m⁻³ d⁻¹</th>
<th>Energy</th>
<th>HRT</th>
<th>carrier</th>
<th>Ref.</th>
<th>P.S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluidised Sandfilter</td>
<td>0.4</td>
<td>n/a</td>
<td>15m</td>
<td>Sand</td>
<td>[200]</td>
<td>Endogenous RAS COD; NO₂⁻ and TAN formation.</td>
</tr>
<tr>
<td></td>
<td>0.86 - 1.33</td>
<td>n/a</td>
<td>0.9- 13m</td>
<td>Sand</td>
<td>Adapt.</td>
<td>Endogenous RAS COD; (Requires pre-treatment for COD); NO₂⁻ and TAN formation.</td>
</tr>
<tr>
<td>Downflow DEN filter</td>
<td>1.6</td>
<td>n/a</td>
<td>n/a</td>
<td>Sand</td>
<td>[201]</td>
<td>COD required;</td>
</tr>
<tr>
<td>Moving bed</td>
<td>2.7</td>
<td>n/a</td>
<td>3h</td>
<td>PP</td>
<td>[202]</td>
<td>Methanol addition;</td>
</tr>
<tr>
<td>Packed bed</td>
<td>2.4</td>
<td>n/a</td>
<td>22m</td>
<td>BG</td>
<td>[203]</td>
<td>Ethanol addition;</td>
</tr>
<tr>
<td>H₂ DEN (electrochemical generation)</td>
<td>0.2</td>
<td>1.17 kWh m⁻³</td>
<td>1h</td>
<td>GP</td>
<td>[27]</td>
<td>Small sludge formation; No COD required; Low H₂ efficiency.</td>
</tr>
<tr>
<td>DEN MFC</td>
<td>0.4</td>
<td>Energy gen.</td>
<td></td>
<td>GG</td>
<td>[53]</td>
<td>Energy generation; COD required at anode; Use of membrane (two chamber system)</td>
</tr>
<tr>
<td>DEN MEC at -0.7 V vs SHE (this study, at 0.5 Ld⁻¹)</td>
<td>0.13</td>
<td>27 kWh Kg N⁻¹</td>
<td>4h</td>
<td>GG</td>
<td>This study Small sludge formation; No COD required; High electron/H₂ uptake efficiency; Buffer dependency; Potential O₂ generation.</td>
<td></td>
</tr>
<tr>
<td>DEN MEC at -0.7 V vs SHE cat potential (this study, at 14.2 Ld⁻¹)</td>
<td>0.12</td>
<td>13 kWh Kg N⁻¹</td>
<td>16m</td>
<td>GG</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

*BG – Brick Granules
*GG – Graphite Granules
*GP -- Graphite Plate
*n/a – data not available
*PP – Polypropylene
1 – Freshwater aquaculture studies
2 – Saltwater aquaculture studies
9.2.1 Buffer capacity considerations

Considering the saltwater culture medium was prepared with 1 g L\(^{-1}\) NaHCO\(_3\) (11.9 mM), approximately 7.3% of this bicarbonate will be converted (at pH 7) to CO\(_2\)/carbonic acid, working as buffer against the pH increased caused by the cathodic reaction. This corresponds to a concentration of approximately 38 mg L\(^{-1}\) CO\(_2\), which is 2.6 times higher than the 15 mg L\(^{-1}\) CO\(_2\) (0.34 mM) suggested for aquaculture waters [12]. However, as discussed in Chapter 8, although this 11.9 mM bicarbonate added is already relatively higher than recommended in aquaculture tanks, it was not enough to maintain pH balance in the cathodic denitrifying biofilm. Thus, buffer capacity as CO\(_2\) – which will be further affected by the pH of the recirculating aquaculture water – might limit cathodic denitrification and further addition of bicarbonate may not be recommended as it will likely increase CO\(_2\) levels to concentrations higher than acceptable for fish production. Interestingly, the nitrifying biological filters commonly installed at a RAS are known to decrease pH and alkalinity of the water, thus this reduced alkalinity could be beneficial for the following denitrification step [204]. Noteworthy, when considering autotrophic nitrification stoichiometry (Equation 2 and Equation 3), it can be seen that 2 protons are produced for each NH\(_4^+\)-N oxidised to NO\(_3^-\)-N. Thus, if nitrate is leaving the nitrifying tank towards the denitrification step, those generated protons can theoretically provide 33% of the protons required for buffering the cathodic reduction of nitrate to dinitrogen gas (Equation 12). Moreover, alkalinity and buffer capacity of the recirculating water leaving the nitrification filter can be re-stored within the denitrification system, which could potentially enable a reduction of bicarbonate addition commonly done in aquaculture for alkalinity restoration [204]. Therefore, it is suggested herein that the cathodic denitrification reactor could be installed after the nitrification step and it is hypothesised that a slightly higher overall recirculation level of the aquaculture stream through the denitrifying reactor would enable low nitrate levels that are well matched with the buffer concentration provided by CO\(_2\) in the recirculating water.
10 Conclusions and Recommendations for future work

This chapter highlights the main conclusions of the different studies carried out during the PhD program. In addition, based on the results exposed in Chapters 5 to 8 and the obtained conclusions, it provides an overview of recommended future work.

10.1 Conclusions

Considering that ammonium formation from nitrate via Dissimilatory Nitrate Reduction to Ammonium may represent an inefficiency for the proposed processes aiming to reduce nutrient loads from water streams, the Objective 1 of this PhD project aimed to understand the possible DNRA as a competitive pathway during cathodic denitrification. The experimental results and analysis presented in Chapter 5 confirmed that this indeed is a possible (competing) nitrate reduction pathway during denitrification in mixed culture cathodic denitrifying biofilms operated at -0.9 V vs SHE. This study also demonstrated that ammonium generation via DNRA pathway is dependent on biofilm age and can decrease over time of operation, likely due to reduced hydrogen availability within the biofilm. The results presented in Chapter 5 stress the importance of having a fully developed biofilm for cathodic reduction of nitrate from contaminated water/wastewaters, in order to avoid unwanted production of ammonium and the consequent retention and/or discharge of nitrogen compounds.

In light of the real need to increase current generation in bioelectrochemical systems (for full scale applications), the Objective 2 focused on strategies (i.e. applying better electrode materials and an enriched electroactive microbial consortia) which could potentially lead to increased electron transfer and denitrification rates. As described in Chapter 6, although increased current generation can be achieved with Vulcan-treated RVC electrodes due to increased surface area available for bacterial growth, bubble formation (due to either the gaseous N₂ end product or unused H₂) may pose an obstacle to the use of this material for denitrification, especially at the low applied cathodic potential (-0.9 V vs SHE). In addition, the use of low cathodic potentials for denitrification can lead to excessive biofilm growth, imposing mass and/or electron transfer limitations. Moreover, the use of an electroactive
inoculum source did not enhance current generation or denitrification rates, and the microbial community has shifted significantly, independently of the inoculum source used.

A novel easy-to-operate (membraneless) upflow setup was proposed as the Objective 3, and operated with graphite granules as both anode and cathode material (at -0.7 V vs SHE) as a polishing mechanism for denitrification from secondary effluents (Objective 3.1 of this PhD project). The technology presents an easy to operate system which was able to perform denitrification to very low concentrations of nitrate (<5 mg L\(^{-1}\)), in the absence of organic matter as energy source and with low conductivity/buffer capacity water stream. The results presented in Chapter 7 showed that both migration and convection play a role in ion transfer in the system when operated with low conductivity water stream. Moreover, although big potential losses especially around the cathode electrode represent an important limitation for the applicability of BES as a polishing mechanism for secondary effluents, the inclusion of full recirculation (both circuits within cathode and anode) can improve electrochemical response and save up to 14% of energy consumption of the system.

As described in Chapter 8, to the best of our knowledge, this is the first study on the use of a BES reactor for denitrification from saltwater streams, indicating the applicability of the upflow membraneless system as a valid alternative to heterotrophic denitrification in saltwater aquaculture streams, either for recirculation or for discharge purposes (Objective 3.2 of this PhD project). When operating at the optimal condition, the proposed system was able to remove nitrate from the synthetic media with minimum ammonium and nitrite accumulation, although nitrous oxide accumulation did occur to some extent. In addition, experimental results detected only small potential losses and indicated that convection did not play a role in ion transfer when treating the seawater medium. However, the performance of the reactor was still enhanced due to a better electrolyte distribution within cathode and anode (but not the gap between them) when using full recirculation regime. Current generation and maximum achievable denitrification rates were shown to be strongly dependent on the effective buffer capacity provided by the bicarbonate system in the medium entering the reactor. Furthermore, the system operated without Cl\(_2\) generation, despite the high salt concentration of the synthetic seawater media. Thus, the
The proposed technology presents technical potential for simultaneous denitrification and partial re-oxygenation of recirculation aquaculture waters, which will improve the quality of the recirculated water (and thus produced fish) and reduce the volume of water exchange from these recirculating aquaculture systems.

### 10.2 Recommendations for future work

An important factor for consideration is the possibility of a shift in microbial community composition over operation time of the BES reactor. An analysis of cathodic biofilm community structure over time is warranted for future work, in order to identify the main drivers and the dynamics of the DNRA process in denitrifying BES. In addition, since N₂O accumulation was considerably higher in the saltwater operated cathode than in the other studies presented herein, further studies would also be necessary to assess the role of the microorganisms identified in the saltwater cathodic denitrifying biofilm. Moreover, further investigations should be carried out in order to minimise the accumulation of this greenhouse gas.

The use of different electrode materials – such as RVC electrodes with bigger pore sizes – may provide bigger surface areas for attachment/growth of biomass and could improve nitrogen removal rates, and further studies should be done in this regard. However, although better performances are generally expected with increased surface areas of 3D electrodes, they may cause an increase in ion transfer losses in the liquid phase. In addition, since our results show that current generation is strongly dependent on buffer capacity of the water stream being treated, we suggest that – in real conditions – the electrode surface area may not be the most important limiting factor in the BES, but buffer capacity itself. Furthermore, as suggested by Jourdin et al., 2015 [199], electrode material may represent the main factor increasing the capital costs of BES. Therefore, although RVC electrodes with bigger pore sizes should be tested in future studies, the excessive growth of biofilm observed at low applied cathodic potential herein indicate that the use of electrode materials such as graphite with relatively fine granules could also be a suitable (cheaper) alternative, with the advantage of enabling an easier removal of bubbles and excess biofilm due to its movable structure.
As previously discussed, potential losses were here demonstrated to occur especially at the cathode electrode. Considering the need to scale up the reactors, studies on the characterization of those potential losses across different depths of the cathode electrode, together with a more detailed analysis of biofilm electroactivity and microbial composition across the cathode bed are highly recommended. Furthermore, although care should be taken in regards to the potential losses across the cathodic depth, using a more specific microbial community which is able to perform direct electron transfer using more positive cathodic potentials, as previously shown [96], will enable further energy savings due to a lower overall achieved cell voltage. Considering the cathodic potential of -0.7 V vs SHE applied in the saltwater study and the theoretical redox potential of +0.82 V for oxygen formation, a cell voltage of 1.52 V with hydrogen formation could be calculated, whereas a cell voltage of 1.0 V would be obtained if direct electron transfer was achieved with the use of the cathodic potential -0.4 V vs SHE. Moreover, by applying more positive cathodic potentials, a reduced biomass formation would be expected. Therefore, these effects should be subject of investigation in future BES studies.

As oxygen was removed from the wastewater feeding the reactor in the present work, further studies should also include the effects of different oxygen concentrations on reactor’s behaviour. As clarified secondary effluent containing nitrate as substrate was used as feed for cathodic reactions, and considering that aerobic tanks require medium to high DO in order to achieve complete nitrification, oxygen will certainly be present in the water stream entering the BES reactor and should play a critical role in the proposed process. It has been previously shown that an adapted cathodic biofilm performing simultaneous nitrification and denitrification is still able to reduce nitrate with dissolved oxygen levels up to 5.73 mg L⁻¹ due to differences in bacterial community structure at a microscale, where organisms present at the outer biofilm layer were able to reduce oxygen concentration before nitrate could be reduced by the denitrifiers located in the inner layers (close to the electrode) [154]. Alternatively, it is hypothesised herein that the effect of oxygen eventually present in real wastewaters may be minimised in the proposed upflow setup by its consumption within initial (lower) portions of the cathode biofilm, thus still enabling denitrification at low oxygen levels at the upper portions of the biofilm.
In regards the anode electrode, the fact that a slightly larger dissolved oxygen concentration was detected at smaller flow rates (longer HRT) supports the hypothesis that oxygen mass transfer from the electrode was limited and led to the formation of bubbles which were not easily dissolved in the water column. Thus, the longer HRT would enable a higher transfer of oxygen from the bubbles formed at the anodic surface towards the liquid phase as dissolved oxygen. Therefore, an improvement of anodic electrode materials, configuration and mixing regimes which enable better transfer/diffusion of oxygen from the electrode to the liquid phase are also strongly encouraged, especially regarding the aquaculture application in which oxygen generation is desired.

Moreover, it is also suggested that tests with an experimental recirculating aquaculture system integrating a denitrifying BES unit should be done to check the effectiveness of the method. Those investigations should include pilot plant tests investigating the evolution of the integrated system since start-up of denitrifying biofilm, following the aquaculture operating cycle (i.e. according to fish growth/fish stock density and water characteristics, in which ammonium and nitrate concentrations are expected to be increasing).
References


28. Aquaculture Regulation in Queensland. 2014, Queensland Competition Authority: QLD.


