Supporting Information for

Bioelectrochemical Denitrification for the Treatment of Saltwater Recirculating Aquaculture Streams

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Supporting Text 1. Preliminary reactor operation

The synthetic medium used for the preliminary reactor operation consisted of 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgSO_4 \bullet 7H_2O$, 0.015 g L^{-1} $CaCl_2 \bullet 2H_2O$, 1 g L^{-1} $NaHCO_3$, 20 mg L^{-1} NO_3^--N and trace elements solution as previously described[1]. At this stage, the reactor was inoculated by feeding it with the effluent from a previously running cathodic denitrifying parent reactor for approximately 2 months, and enriched in continuous mode.

Supporting Text 2. ANOVA: denitrification rates between 2 L/d and 3 L/d flow rates

Table S1 – Calculated denitrification rates at 3 and 2 L/d flow rates.

Flow rate	3 L/d	2 L/d
DEN rate (complete) kg N m^3 d^{-1}	0.12	0.14
	0.12	0.14
	0.13	0.12
	0.14	0.12

Table S2 – Summary of data used to carry out the ANOVA test using the excel data analysis Anova: Single Factor function.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
3 L/d	4	0.508427	0.127107	9.38E-05
2 L/d	4	0.519754	0.129938	9.63E-05

Table S3 – ANOVA Results output.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.6E-05	1	1.6E-05	0.168707	0.695529	5.987378
Within Groups	0.00057	6	9.51E-05			
Total	0.000586	7				

Supporting Text 3. Operational costs comparison: BES versus sand-filters

Considering the use of sand(bio)filters as a competing technology for denitrification in aquaculture systems, and considering negligible energy consumption for operation of pumps of both sand filters

and BES, then the operational costs of both systems could be compared as the amount of methanol required for heterotrophic denitrification in sand filter and the amount of electricity required for autotrophic denitrification in 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 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 eventual oxygen consumption). Therefore, when considering that a ratio of at least 4/1 (kg methanol/ kg N) is required [2], then the actual cost would be doubled to approximately AUD$2.00 per kg NO_3^- -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 for industries in Australia as being approximately AUD$0.15 per KWh, then the operational cost for nitrogen removal via autotrophic BES denitrification (at 27.3 ± 6.5 kWh kg N^{-1}) presented herein is approximately AUD$4.46 per Kg N removed, which is currently twice as much as the calculated operational cost for the operation of sand filters.

Supporting Text 4. Saltwater adapted cathodic denitrifying biofilm: microbial community composition assessment

Methodology

After completion of all experiments, microbial community analysis was done by collecting biofilm sample using a sterile syringe and needle. The biomass sample was collected at different portions of the cathode bed (lower, mid and upper portions of the cathodic granules) and provided to the Australian Centre for Ecogenomics (ACE) for DNA extraction and 16 rRNA Amplicon sequencing as previously described. [3]

Results

Analysis of the microbial community structure via sequencing of the 16S rRNA gene are summarized in Figure S1, 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 S1).

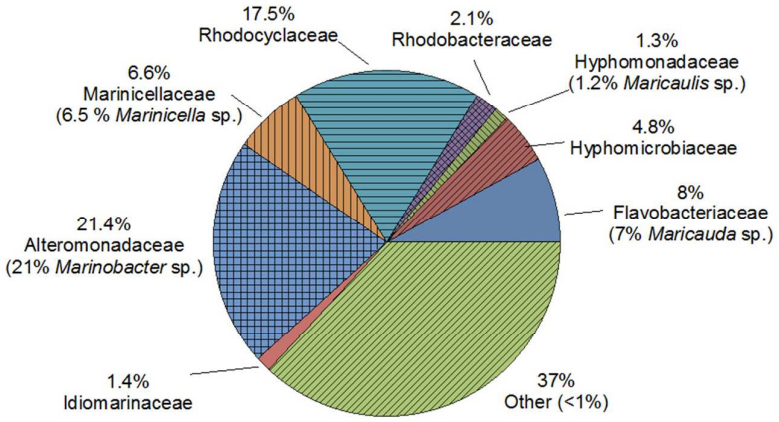

Figure S1 – Microbial community structure (main Families and Genera, and their relative abundance) detected in the cathodic denitrifying biofilm treating synthetic seawater aquaculture stream.

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.[4, 5]

Although some organisms of the genus *Marinobacter* (family Alteromonadaceae) are typically known to be heterotrophs,[4] many species such as *M. subterrani* and *M. aquaeolei* have been reported able to perform Fe (II) oxidation.[6, 7] 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.[8] *S*pecies such as *Marinobacter hydrocarbonoclasticus* are well known denitrifiers[4] and have been previously enriched in denitrifying reactors using Hydrogen as electron donors.[9] Similarly, some strains of *Maricaulis* sp. (family Hyphomonadaceae) are also known to reduce nitrate,[10] 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.[11] Therefore, microorganisms belonging to the *Marinobacter* genus and to the families Hyphomicrobiaceae and Hyphomonadaceae are likely the key autotrophic denitrifiers in the present study.

A few *Marinicella* sp. (an unclassified genus belonging to the class Gammaproteobacteria) have firstly been reported as an aerobic bacteria unable to reduce nitrate.[12] 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.[13] Similarly, although a recent study reported some members of *Maricauda* genus (Flavobacteriaceae) as unable to perform denitrification,[14] a few species from this family (i.e. *Maribacter* sp.) were previously reported as nitrate reducers,[15] or able to perform complete denitrification (i.e. *Flavobacterium banpakuense*).[16] Furthermore, other species from this family have been previously detected in engineered experimental soil columns performing denitrification[17], as well as in natural seawater environments.[18, 19] 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. Thus, further studies should be necessary to assess the role of these organisms in the cathodic denitrifying biofilm in the present study.

M. hydrocarbonoclasticus (specie closely related to the *Marinobacter* OTUs recovered in the present work) is known to possess the enzime nitrous oxide reductase (N_2OR),[20] indicating they are likely playing a role on N_2O reduction in our system. Several genera within the families Rhodobacteraceae and Rhodocyclaceae (2.1% and 17% relative abundance, respectivelly) are known to reduce nitrate to nitrite.[11] However, only a few genera belonging to those families were reported to proceed complete denitrification, including *Paracoccus* sp. (Rhodobacteraceae) and *Thauera* sp. (Rhodocyclaceae),[11, 21] 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. Therefore, further investigations should be done to determine the reasons for, in order to minimize the formation of this greenhouse gas.

Although a few genera of the families Rhodobacteraceae (Alphaproteobacteria)[22, 23] and Rhodocyclaceae (Betaproteobacteria) [22, 24-26] 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 presented here.

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