TREATMENT OF POLLUTED SALINE AND NON-SALINE GROUNDWATER THROUGH HYDROGENOTROPHIC DENITRIFICATION.

Yashika G. De Costa (The University of Auckland), Yang Wu (Peking University, China), Dr. Ke Yu (Peking University, China) and Dr. Wei-Qin Zhuang (The University of Auckland).

ABSTRACT (500 WORDS MAXIMUM)

Hydrogenotrophic denitrification (or hydrogen-oxidising autotrophic denitrification) is an alternative method used to biologically remove nitrate and nitrite from polluted waters. Compared to conventional heterotrophic denitrification, which requires organic carbon as an electron donor, this process uses hydrogen gas as an electron donor to reduce nitrate/nitrite to nitrogen gas. In this regard, hydrogenotrophic denitrification bears a few engineering advantages for water treatment. For example, hydrogen gas is benign to humans, and hydrogen-utilising microbes have a low biomass yield in general.

The use of hydrogenotrophic denitrification may prove to be useful for New Zealand, which is experiencing increased groundwater pollution due to intensifying unsustainable agricultural activities and dairy farming. Furthermore, due to rising sea levels, seawater intrusion has become an added concern for coastal cities. Seawater intrusion increases the salinity of groundwater, which can hinder the activities of conventional biological groundwater treatment processes. It is evident that to use hydrogenotrophic denitrification to treat groundwater in New Zealand the process needs to be effective in both saline and non-saline conditions.

In this study indigenous hydrogenotrophic denitrifiers were enriched from different sources taken from saline and non-saline environments, respectively. The study proved to be successful, yielding a 97% nitrate removal in non-saline conditions with an average removal rate of 70±2 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$. In saline conditions (4% salinity) a 92% nitrate removal was achieved, with an average removal of 52±2 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$. Furthermore, 16S rRNA gene identification of the non-saline samples revealed that members of the genus *Thauera* were predominant in the non-saline enrichment culture with an abundance of 18% of the total microbial community. Many cultured species of the genus *Thauera* are known denitrifiers. In particular, some are capable of carrying out hydrogenotrophic denitrification as well as denitrification in aerobic environments.

KEYWORDS

Hydrogenotrophic denitrification, autotroph, nitrate pollution, saline water

PRESENTER PROFILE

Mr. Yashika G. De Costa is a PhD student at the University of Auckland (UoA) under the supervision of Dr. Wei-Qin Zhuang. Mr. De Costa completed his honors degree in Civil Engineering also at the UoA. His focus as a researcher is on Environmental Microbiology with particularly interests in bioremediation and nutrient removal in saline environments.

1 INTRODUCTION

Excess levels of nitrate or nitrite in drinking-water sources can be toxic to humans, causing issues such as gastric cancer and methemoglobinemia for infants (Kapoor and Viraraghavan, 1997; Kim et al., 2002). The current recommendation for nitrate-nitrogen (NO_3^--N) in drinking water stands at 10 mg L^{-1}, as proposed by the World Health Organisation (World Health Organization, 2011). Agricultural activities and industrial wastes that release nitrogen into the environment threaten this drinking-water standard. For example, the invention of the Haber-Bosch process, producing NH_3 from N_2, has introduced copious amounts of reactive nitrogen into the environment, amounting to a ten-fold increase compared to the values from 40 years ago (Van Hulle et al., 2010).

Particularly concerning in New Zealand is the increase of nitrogen-based fertiliser applied to topsoil, which percolates through the soil and ends up in groundwater sources, accumulating to higher levels as farming continues (Ju et al., 2017). Being an agricultural intensive country, New Zealand's dairy industry is valued at approximately NZ$17 billion (NZIER, 2018). This industry, however, has caused a 25% increase in the use of nitrogen-based fertilisers since 2002, leading to an estimated 29% increase in agricultural nitrogen leaching (Ministry for the Environment and Statistics New Zealand, 2015). Studies from 200 identified groundwater sources have shown a strong correlation between the expansion of the dairy industry from 2002-2016 and an upward trajectory of NO_3^--N concentrations in groundwater from 2005-2014 (Ministry for the Environment and Statistics New Zealand, 2015)(Figure 1)

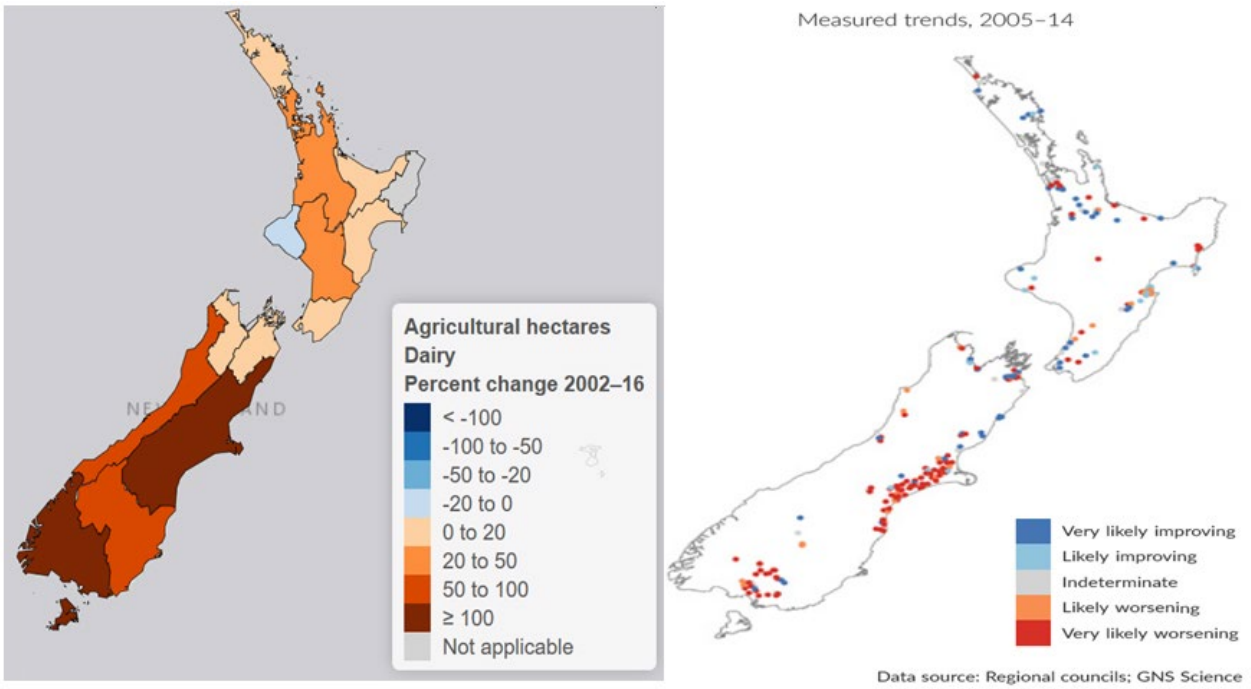

Figure 1: Agricultural use and groundwater pollution in New Zealand (Ministry for the Environment and Stats NZ, 2019).

Furthermore, with the advent of climate change and rising sea-levels, the issue of sea water intrusion becomes an added concern for groundwater pollution. Specifically in New Zealand, where 150 of the identified groundwater sources are situated near the coast (NZ, 2015). Generally, the treatment of saline water occurs through ion exchange (Ghafari et al., 2010), creating a concentrated brine solution which needs further treatment before disposal. The brine to be treated typically exhibits elevated salinity

(generally, 4%-26%), which hinders the usual biological nitrate removal process. This study, therefore, investigated the use of indigenous microbes to perform hydrogenotrophic denitrification – in both saline (low salinity-brine) conditions (4%) and in non-saline conditions.

1.1 HYDROGENOTROPHIC DENITRIFICATION

Hydrogenotrophic denitrification (hydrogen-oxidising autotrophic denitrification) is an effective biological process that relies on autotrophic microbes to reduce nitrate/nitrite by using H_2 as an electron donor. It is a promising alternative to conventional heterotrophic denitrification used in wastewater treatment plants, which requires either an exogenous organic carbon electron donor (i.e. methanol), or endogenous respiration to facilitate the process. In general, autotrophic denitrification has some advantages over the conventional heterotrophic denitrification. Specifically, cost reductions from alleviating the need for additional organic carbon, and the abridged scale of sludge processing and disposal (Cord-ruwisch et al., 1988). In wastewater treatment plants, however, the use of heterotrophic denitrification is employed due to its high efficiency of reduction, general availability of natural organic substrates, and ease of use (Zhao et al., 2011). Notwithstanding, for conditions such as groundwater treatment, autotrophic denitrification can be considered, being cheaper and not resulting in secondary organic carbon pollution.

Autotrophic denitrification can occur by using either sulphur, iron, or hydrogen gas as electron donors, of which the use of sulphur and hydrogen is the most widely regarded (Kapoor and Viraraghavan, 1997). Comparisons of conventional organic substrates used in heterotrophic denitrification and sulphur/hydrogen based autotrophic denitrification can be seen in Table 1. As an autotrophic process, the use of sulphur as a substrate is cheaper, however the lower nitrate removal and by-products (which lowers pH) make hydrogen-based denitrification more attractive (Zhao et al., 2011). The hydrogen-based process also coincides with the speculated growth of a hydrogen utilizing economy as an energy source in the future (Züttel et al., 2010).

Table 1: Heterotrophic and Autotrophic denitrification efficiencies (Park and Yoo, 2009)

Substrate	**Cost** $/kg substrate	**Consumption** kg substrate/kg N-NO_3^-	**Cost of denitrification** $/kg N-$NO_3^-$	**Nitrate Removal rate** kg-N $m^{-3}d^{-1}$
Methanol	0.92	2.08-3.98	1.8-3.6	1-27
Acetic Acid	2.21	2	7.36	-
Acetate	1.67	2.7	4.37	0.6-1
Ethanol	1.10	3.5	2.2	0.4-1.2
Cotton	0.53	2.8	1.48	0.36
Sulphur	0.1	2.5	0.25	0.05
Hydrogen	**2.2-3.1**	**0.43**	**0.95-1.3**	**0.5-2.4**

In New Zealand, a study of hydrogenotrophic denitrifiers using indigenous seeds provides two essential benefits; 1) Biological remediation using indigenous strains have shown to outcompete foreign strains with environmental variations (Iwabuchi et al., 1998), and 2) New Zealand has strict bio-security controls which makes it difficult to import foreign strains to be used in large-scale engineering processes. More importantly the study promotes a more sustainable approach of denitrification, which also counteracts and minimises the effects caused by climate change. Therefore, the main objectives of the study are:

1) Enriching hydrogenotrophic denitrifiers using indigenous seeds.

2) Investigating nitrate removal efficiencies of the enrichment cultures.

3) Understanding the diversity of hydrogen-utilising microbes within the enriched hydrogenotrophic denitrifying cultures.

2 EXPERIMENTATION PROCEDURE

2.1 REACTOR SETUP AND OPERATION

The enrichment of hydrogenotrophic denitrifiers were carried out in batch experiments, in glass serum bottles (160 mL) with a working volume of 100 mL. Seed sludge for the non-saline culture was obtained from a local wastewater treatment plant (WWTP) in Auckland, New Zealand. For the saline culture, marine sediment samples were collected from a local beach. The composition of the Mineral Salts Medium (MSM) and trace elements solution can be found in Table 2. Sodium bicarbonate, $NaHCO_3$ (1.1 g/L) was added to the medium as both a pH buffer and to serve as an inorganic carbon source (HCO_3^- is naturally found in groundwater). The final pH of the culture medium was 7.4±0.2. The bottles were then sealed with butyl rubber stoppers and vacuumed for one minute to remove the oxygen-contained headspace. Hydrogen gas was then injected into each bottle and pressurised to 10 psi under room temperature. The H_2 gas was replenished periodically depending on its utilization. Triplicated serum bottles were prepared in non-saline conditions and in saline conditions (adjusted to 4% salinity using sodium chloride, NaCl). Sodium nitrate ($NaNO_3$) (≥99% purity, Sigma), was injected into the serum bottles periodically, the loading was regularly adjusted to match the enrichment culture's performance. The enrichment cultures have been maintained in the lab for two years to date.

Table 2: Culture medium composition used in this study (Zhuang et al., 2011)

Mineral Salts	Concentration (g/L)	Trace elements stock	Concentration
NaCl	1.0	HCl (25% solution)	20 µL
$MgCl_2.6H_2O$	0.5	$FeCl_2.4H_2O$	3 mg/L
KH_2PO_4	0.2	$CoCl_2.6H_2O$	0.38 mg/L
NH_4Cl	0.3	$MnCl_2.4H_2O$	0.2 mg/L
KCl	0.3	$ZnCl_2$	14 µg/L
$CaCl_2.2H_2O$	0.015	H_3BO_3	1.2 µg/L
$NaSO_4$	0.05	$Na_2MoO_4.2H_2O$	7.2 µg/L
		$NiCl_2.6H_2O$	4.8 µg/L
		$CuCl_2.2H_2O$	0.4 µg/L

2.2 ANALYTICAL MEASUREMENTS

Concentrations of nitrite and nitrate were measured according to Standard Methods 22nd Edition (Rice et al., 2012), 4500-NO_2^- Colorimetric Method and 4500-NO_3^- Ultraviolet Spectrophotometric Screening Method. A UV-Vis spectrophotometer (UV-2700 SHIMADZU, Japan) was used for the measurements. pH was measured using an HACH HQ40d portable meter (HACH).

2.3 BATCH KINETIC ANALYSIS

Batch cultures were conducted to assess the growth and denitrification performance of both saline and non-saline enrichment cultures. A portion of 10 mL from each enrichment culture was aseptically transferred into 90 mL fresh culture media (100 mL working volume). To minimise the effect of O_2 contamination when preparing the batch cultures, the media was first degassed for 30 minutes, then, transferred and kept in an anaerobic chamber with a H_2:CO_2:N_2 atmosphere ratio of 5:5:90, overnight. All other conditions were kept identical to the original enrichment. Samples were taken at three-hour intervals for the measurement of NO_2^- and NO_3^- concentrations and cell numbers, over the course of the experiment. The absorbance at a wavelength of 600 nm was measured

as an indication of the biomass growth and to determine the mean growth rate of the microbial community (i.e., the time taken for biomass to double).

2.4 DNA EXTRACTION AND 16S rRNA GENE SEQUENCING

Genomic DNA samples from the enrichment culture was extracted using the DNeasy PowerSoil Kit (Qiagen). The extracted DNA was eluted with 10 mM Tris-HCl (pH 8). A Nanodrop 8000 spectrophotometer (Thermofisher) was used to assess the purity and quantity of extracted DNA before being stored at -20°C and sent for sequencing. For PCR amplification, the hypervariable V4 region of the bacterial 16S rRNA gene was amplified using universal primers. All PCR reactions were carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's procedures. The library quality was assessed on a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina MiSeq platform and 250 bp paired-end reads were generated.

2.5 DATA PROCESSING

Data processing of 16S rRNA gene sequencing involved analysing abundance and diversity of microbial populations and developing a phylogenetic tree accordingly. The phylogenetic tree was constructed by the neighbour-joining method through the MEGA 7 program (Molecular Evolutionary Genetics Analysis Software, Version 7.0). The obtained 16S rRNA gene sequences were compared to available 16S rRNA gene sequences in GenBank using the NCBI Blast program.

3 RESULTS AND DISCUSSION

3.1 NON-SALINE AND SALINE KINETICS

Overall, for the non-saline enrichment culture, 98% removal of nitrate was achieved over the course of a 28-hour incubation (Figure 2). This corresponds to an average removal rate of 70±2 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$, with a lag phase of seven hours and a peak doubling time of four hours. At its peak a removal rate of 195±2 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$ was achieved. Lag phases that occurred in the culture may be a result of transferring microbes into fresh media causing a depletion of essential constituents, as cells attempted to resynthesize any lost constituents not contained in the original media (Berlanga, 2010). Furthermore, trace O_2 contamination during transfers of the culture may have inhibited the activity of denitrifying-bacteria.

The removal rates achieved in this study were comparable to results obtained by Smith, Ceazan, & Brooks, (1994) who also achieved an average removal of around 70 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$. Similarly, it performed better than a study conducted by Lu & Gu, (2008), who obtained peak removal rates of 155±1 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$. It should be noted that Lu & Gu, (2008) utilised gas permeable membranes in their reactors to improve hydrogen mass transfer, hence their improved removal rates. This is an area that we will also explore in future studies with our enrichment culture.

For the saline enrichment culture, 92% nitrate removal was achieved over a period of 48 hours, corresponding to an average removal rate of 52±2 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$, a lag phase of 15 hours and a peak doubling time of five hours (Figure 3). The peak removal rate was 135±2 mg NO_3^--N $L^{-1}{\cdot}d^{-1}$. This was better than a study conducted by Sahu, Conneely, Nüsslein, & Ergas, (2009) who achieved only 30% removal with a 53 hr batch experiment under 1.25% salinity. However, it should be noted that their study employed a membrane reactor with a higher influent nitrate concentration (1000 mg NO_3^--N L^{-1}). The higher nitrate influent concentration in their study resulted in nitrite accumulation (which was not evident in this study Figure 3) and may have caused inhibition for further nitrate removal.

In that respect, it can be noted that the required water resource regulations of 10 mg NO_3^-N L^{-1} were achieved in both saline and non-saline conditions. It should also be noted that in terms of biomass growth, in the saline culture there was a 45% increase in the OD_{600} absorbance per unit of substrate consumed (mg NO_3^--N L^{-1}). Overall, it was demonstrated that the system could be used under both non-saline and high salinity conditions (i.e. brine up to ~4% salinity) for denitrification, albeit exhibiting a slightly slower performance in saline conditions.

3.2 MICROBIAL COMMUNITY ANALYSIS

After a 10-month enrichment, a functional stable microbial community was taken from the non-saline culture for microbial diversity analysis. 16S rRNA gene identification yielded 755 operational taxonomic units (OTUs), which were used to determine the dominant phyla in the microbial community; *Gemmatominadetes* (16%), *Bacteriodetes* (17%), *Proteobacteria* (24%) and *Firmicutes* (13%). Among which, the genus *Thauera*, belonging to the *β*-proteobacteria class accounted for 18% of the total population. The obtained sequences were compared to the NCBI database, to check for any closely related species. Out of the five most abundant OTUs only two had been previously identified as denitrifiers (Table 3). One OTU identified as belonging to the genus *Thauera* showed a 100% identity to *Thauera mechernichensis,* while another OTU identified as *unclassified_Rhodobacteraceae,* showed a 99.6% relation to *Defluviimonas pyrenivorans.* It should be noted that only 250 base pairs of the V4 region from the 16S rRNA gene

were used as comparisons. The remaining three most abundant OTUs had no closely related culturable species (84%-90% identification is insignificant to make a comparison).

Some *Thauera* species have also been identified in several denitrifying microbial communities previously. A study conducted by Mao, Xia, & Zhang, (2013) on the enrichment of hydrogenotrophic denitrifiers also identified *Thauera* as the dominant genus with an overall abundance of 56%. Their experiment achieved peak removal rates of 100 mg NO_3^--N $L^{-1} \cdot d^{-1}$. The slightly lower removal rates achieved compared to this study, potentially indicate the presence of a more unique microbial consortium in the present cultures.

Furthermore, *Thauera* is known for carrying versatile metabolisms, especially *Thauera mechernichensis* which was determined to be capable of aerobic denitrification (Scholten et al., 1999). This flexibility may prove useful in terms of performance in conditions such as groundwater, which can have dissolved oxygen at concentrations that are inhibitory to conventional denitrification processes.

The saline enrichment culture's microbial population analysis is currently being conducted. It would be interesting to see if *Thauera*-like microbes are still present in the saline environment and to discover the microbial diversity of the enrichment culture. A recent study conducted by Li, Li, Liu, Zheng, & Shapleigh, (2018) found that as salt was introduced into the reactor the relative abundance of *Thauera* decreased significantly from 20% to 5% abundance at 5% salinity. Information like this will allow us to identify and understand species that are more saline tolerant to design and achieve higher denitrification rates in future water treatment processes.

Table 3: Abundant OTUs of the non-saline culture and NCBI related species

Species	Abundance (%)	Most-closely related culturable species	Known Denitrifiers
Unclassified_Rikenellaceae	15.6	*Poryphyromonas pogonae* (86.2%)	-
Thauera	**14.6**	***Thauera mechernichensis*[1] (100%)**	+
Unclassified_Gemmatimonadetes	12.3	*Longimicrobium terrae* (84.8%)	-
Unclassified_Anaerolineaceae	5.6	*Ornatikinea apprima* (90.5%)	-
Unclassified_Rhodobacteraceae	**4.9**	***Defluviimonas pyrenivorans*[2] (99.6%)**	+

[1]Scholten et al., 1999
[2]Zhang et al., 2018

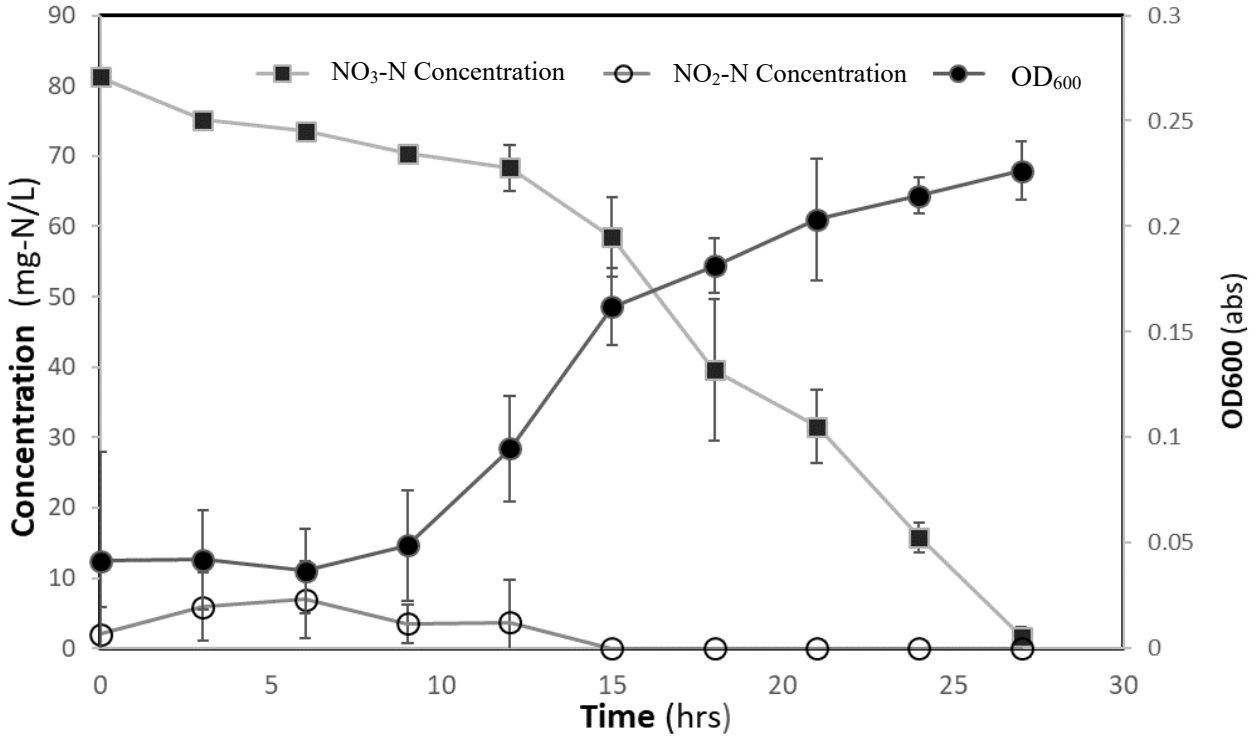

Figure 2: Kinetic study of hydrogenotrophic denitrification in non-saline conditions (<0.2% salinity)

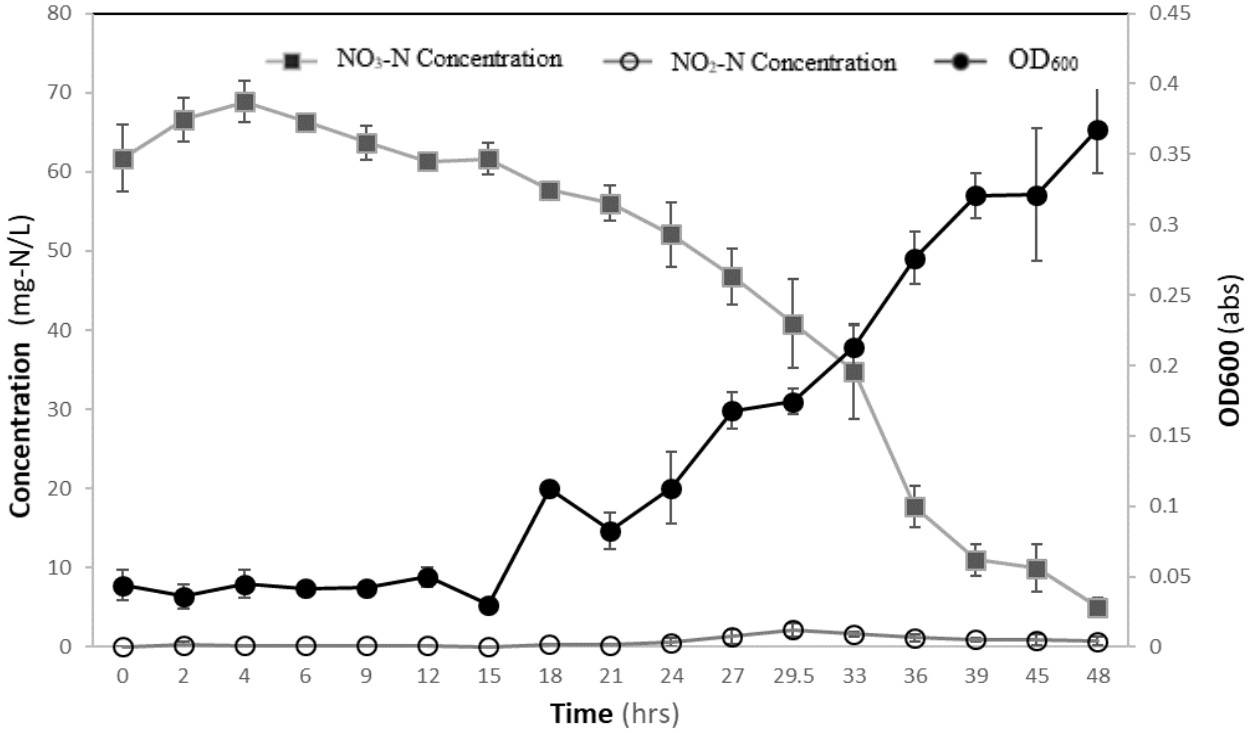

Figure 3: Kinetic study of hydrogenotrophic denitrification in saline conditions (4% salinity)

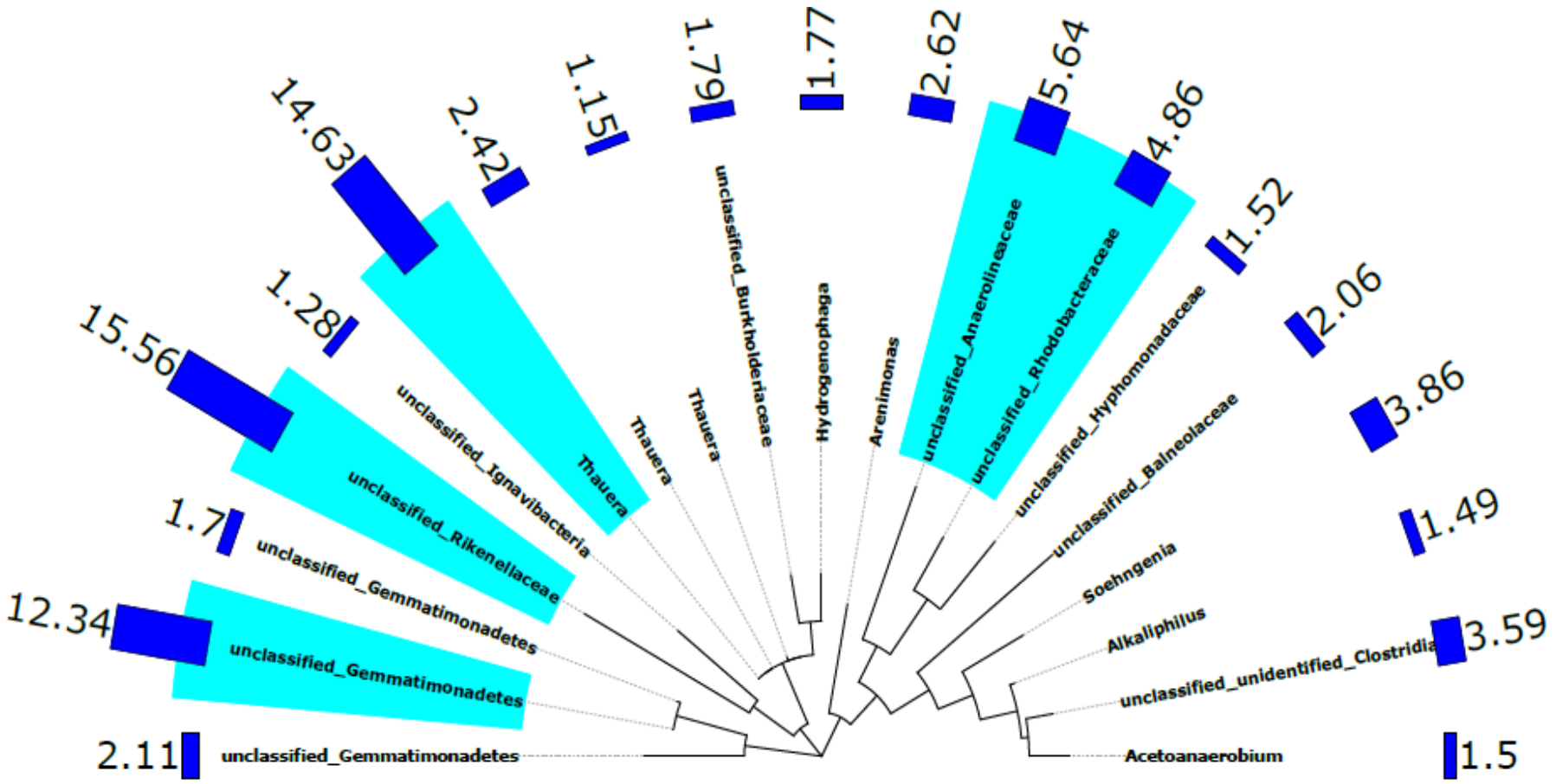

Figure 4: Phylogenetic tree of the non-saline hydrogenotrophic denitrification enrichment culture taken at the 10th months from the culture first started.

4 CHALLENGES AND FUTURE DEVELOPMENTS

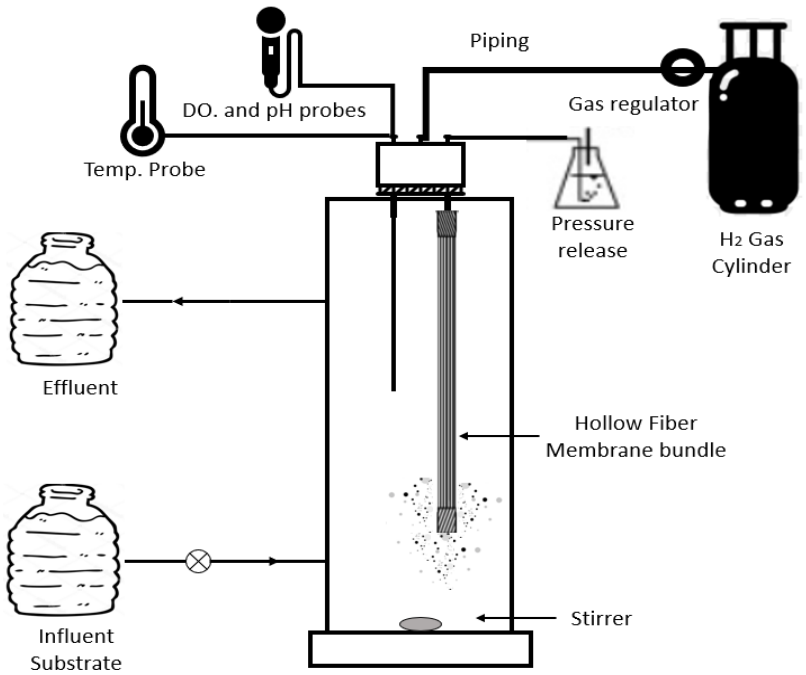

Figure 5: Schematic of a bench-scale membrane bioreactor designed to carry out a continuous flow hydrogenotrophic denitrification process

The next stage of this study will look at using the enriched cultures in a continuous flow reactor (Wu et al., 2019), where H_2 gas will be supplied into the system using a hollow fibre membrane module.

One of the key issues identified with hydrogenotrophic denitrification is the low solubility of H_2 in water and its potential explosive risk (Karanasios et al., 2010). Both issues can be minimised using membrane bioreactors, (Figure 6) (Mo et al., 2005). Membranes (i.e. hollow fibre membranes) can be used to transfer hydrogen to large catchments, which can help avoid hydrogen leakage and increase solubility through high surface area diffusion of H_2 into the environment.

In the case of in-situ groundwater treatment in the field, the membrane module can be placed downstream to allow groundwater to flow through the modules and be treated (EPA, 2013), in a similar manner to a continuous flow module as designed in the lab.

5 CONCLUSION

Nitrate reduction was achieved in batch reactors in both saline (4%) and non-saline conditions. For the non-saline hydrogenotrophic culture a nitrate removal of 97% was achieved. Whilst for the saline enrichment (4%) a nitrate removal of 92% was achieved. In both cases however, the water regulation of 10 mg NO_3^--N L^{-1} was achieved, allowing for safe consumption in non-saline conditions, and safe disposal of saline brine.

The investigation conducted in this study proved the existence and feasibility of hydrogenotrophic denitrification in both saline and non-saline environments in New Zealand. Furthermore, microbial community analysis revealed the abundance of *Thauera* in the community. *Thauera* which is capable of growth in aerobic conditions, will be an interesting topic of study for hydrogenotrophic denitrification of groundwater. Further research to develop this idea would involve shifting the laboratory experiments from batch studies to a continuous flow system to simulate groundwater environments. Lastly, developing a better understanding of the microbial communities in the saline culture in order to analyse a more saline tolerant community will be useful for efficient treatment in brine waste.

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