Nitrogen removal and ammonia-oxidising bacteria in a vertical flow constructed wetland treating inorganic wastewater

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ABSTRACT: Nitrogen removal performance and the ammonia-oxidising bacterial (AOB) community were assessed in the batch loaded 1.3ha saturated surface vertical flow wetland at CSBP Ltd, a fertiliser and chemical manufacturer located in Kwinana, Western Australia. From September 2008 to October 2009 water quality was monitored and sediment samples collected for bacterial analyses. During the period of study the wetland received an average inflow of 1109m^3/day with NH_3-N = 40mg/L and NO_3-N=23mg/L. Effluent NH_3-N and NO_3-N were on average 31mg/L and 25mg/L respectively. The overall NH_3-N removal rate for the period was 1.2g/m^2/d indicating the nitrifying capacity of the wetland. The structure of the AOB community was analysed using group specific primers for the ammonia monooxygenase gene (*amoA*) by terminal restriction fragment length polymorphism (T-RFLP) and by clone libraries to identify key members. The majority of sequences obtained were most similar to *Nitrosomonas* sp. while *Nitrosospira* sp. was less frequent. Another two vertical flow wetlands, 0.8ha each, were commissioned at CSBP in July 2009, since then the wetland in this study has received nitrified effluent from these two new cells.
Keywords: *amoA*, constructed wetland, nitrification, nitrogen removal T-RFLP, vertical flow.

Introduction

Vertical flow constructed wetlands (VFCWs) have proven to be an efficient alternative in the treatment of ammonium contaminated wastewater (Brix and Arias, 2005). Due to their oxygen transfer capabilities VFCWs can harbour a diverse nitrifying community in their filtering layer (Tietz et al., 2007). Bacterial nitrification plays a major role in ammonium removal from wastewater therefore characterising the nitrifying bacterial communities and their distribution within a wetland system is important.

Ammonia oxidation is thought to be the main limiting factor for the conversion of ammonia to nitrate in most systems as nitrite concentrations in the environment are usually low (De Boer et al., 1990). Knowledge of the ammonia oxidising bacterial (AOB) community structure and composition may contribute to process optimisation of systems relying on conventional nitrification as first step for nitrogen removal. It is known that systems with higher diversity are less prone to failure (Purkhold et al., 2000) however little research has focused on microbial analysis of wetlands (Faulwetter et al., 2009).

Nitrifying bacterial communities have been characterised in many engineered systems such as membrane bioreactors (Wittebolle et al., 2008), sequencing batch reactors (Wittebolle et al., 2008), activated sludge plants (Siripong and Rittmann, 2007), VFCWs (Tietz et al., 2007) and chloraminated drinking water supply plants (Regan et al., 2002). AOB are ubiquitous and have been isolated from soils, fresh, brackish and marine waters and sediments, salt lakes and other environments (Kowalchuk & Stephen, 2001; Koops et al., 2006).

The objective of this study was to assess nitrogen removal performance and to characterise the AOB community in the superficial sediment layers of the wetland at CSBP Ltd., a chemical and fertiliser manufacturer located in Kwinana, Western Australia. The potential contribution of ammonia-oxidising archaea (Crenarchaeota) and heterotrophic nitrifiers in ammonia removal in the wetland is recognised, their characterisation however was not within the scope of this study.

Materials and Methods

Wastewater and wetland description

Cooling tower blowdown and stormwater runoff from CSBP's Kwinana site is directed to its wastewater containment system. The inorganic wastewater has an average pH=7.5, COD = 50mg/L and a COD:N ratio $\leq$ 1. Most of the influent nitrogen is in the form of NH_3-N. In 2004 CSBP constructed a pilot nutrient stripping wetland (wetland cell 1) to treat part of this wastewater prior to discharge. This wetland was designed to treat up to 500m^3/day of wastewater with a targeted 50% nitrogen removal. In order to handle larger volumes of wastewater produced two new batch-fed VF wetlands (namely cells 2 and 3), 8000m^2 each, were incorporated into the treatment train in July 2009. Cells 2 and 3 were placed between the containment pond and wetland cell 1 so from August 2009 wetland cell 1 started receiving nitrified effluent from cells 2 and 3. This study analysed the performance of wetland cell 1 from September 2008 to October 2009.

CSBP's wetland cell 1 is 135m long x 95m wide planted with *Schoenoplectus validus* and has a 1m deep fine sand substrate. At the time of construction a 10cm layer of woodchips was placed in the middle of the sand substrate with the goal to provide a carbon source for denitrification. The influent distribution pipe is located centrally on the surface of the sand and the effluent drainage pipes located underneath the sand column on the surface of a plastic liner, drainage pipes are covered with gravel and wrapped in geotextile fabric. The system is a saturated surface VFCW operated in a sequencing batch mode with approximate cycle times of 12hr –feed, 0 to 24hr –stay, 12hr –drain. The sand medium is kept constantly saturated with the water level varying between the sand surface and 0.3m above it (**Figure 1**). The wetland is never fully drained and draining stops when the water level reaches the surface of the sand, at that time filling of another batch starts. Batch volumes and residence times are highly variable due to uneven wastewater production and storm events. Batches ranging from 31 to 5333m^3 were recorded during this study period with an average inflow of 1109m^3/day. With a total holding volume of 7695m^3, an average theoretical hydraulic retention time (HRT) of 6.9 days can be assumed. Heavy metal removal performance for this wetland has been described by Domingos et al. (2009).

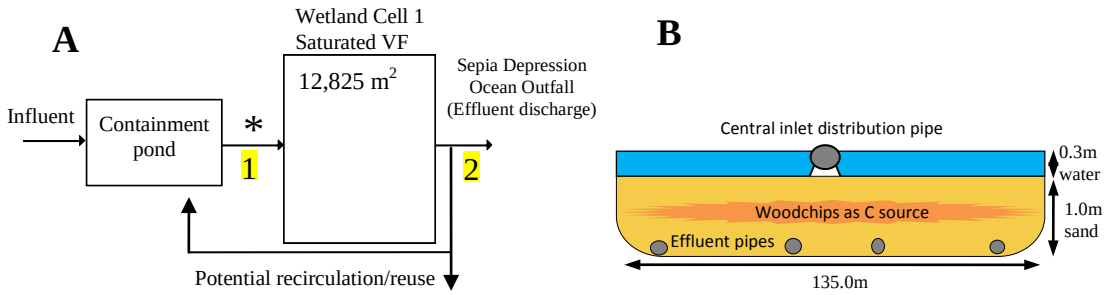

Figure1. **A**: Schematic view of the treatment train at CSBP Ltd. The asterisk between the containment pond and cell 1 indicates where the new cells were built (July 2009). 1 and 2 indicate water sampling points. **B**: Cross-section view of the wetland.

Water, sediment and microbial sampling and analyses

The wetland is equipped with flow meters and automated composite samplers in the inlet and outlet pipes. Samples are analysed for pH, conductivity, ammonia, nitrate, ammonia + nitrate= total nitrogen (TN), total phosphorous (TP) and a wide range of metals according to Standard Methods (APHA, 1998) in the CSBP laboratory.

For microbial analysis samples were collected from the very surface layer, <1cm deep, (sludge) and from a 10 cm depth where the sediment consisted of clear sand (sand). Sediment was taken by inserting a graded plastic pipe to the desired depth and then collecting approximately 10.0g of sludge and sand material in zip-lock bags. Sampling was conducted at 3 locations in the wetland in August 2008, September 2008, February 2009 and August 2009, samples from the same depth were grouped as sludge or sand. DNA was extracted with the use of UltraClean® soil DNA isolation kit (Mobio Laboratories, CA.) according to the manufacturer's instructions, eluted in water and stored at -20°C until use.

Polymerase Chain Reactions (PCRs)

The AOB specific primer pair *amoA*-1F (5'-GGG GTT TCT ACT GGT GGT) and *amoA*-2R (5'-CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997) targeting a 491 base pair (bp) region of the *amoA* gene was used in the PCR. Amplifications were performed in 25µL reaction volumes for cloning purposes and 50µL reactions for T-RFLP analysis. Reactions contained 10 to 20ng of template DNA, 1x PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of mixed dNTPs, 0.5µM of each primer 1.0 U of *Taq* DNA polymerase (Promega, Madison, Wi). PCR cycles used have been described by Siripong and Rittmann (2007) with annealing at 55°C. PCR products of the expected size were purified with the Wizard SV Gel and PCR clean up kit (Promega, Madison, Wi), for further use.

Clone library

Cloning reactions and transformations were performed according to the instructions in the PGEM-T easy Vector System and JM109 competent cells (Promega, Madison, Wi). A total of 136 positive clones from the August and September 2008 samples were selected for the amplification of the *amoA* gene and subsequent digestion with HaeIII and AluI RE according to the manufacturers' instructions. Digested products

were electrophoresed and visualised in 3% agarose gels. Different restriction fragment patterns were assigned to different operational taxonomic units (OTUs).

T-RFLP

The *amoA*-1F- 5'FAM labelled primer was used to generate PCR products for T-RFLP. Purified PCR products were digested with Alu I, HaeIII and TaqI restriction enzymes (RE) according to the manufacturers' recommendations (Promega, Madison, Wi). One µl of each digest was added to 0.25 µl LIZ600 marker (Applied Biosystems, CA) and 9 µl Hi-Di™ formamide (Applied Biosystems, CA) and fragment analysis performed in an Applied Biosystems 3730 DNA sequencer (SABC, Murdoch University). Resulting profiles were analysed using Genemapper software (Applied Biosystems, CA). T-RFLP profiles were normalised according to the constant percentage threshold (Sait et al., 2003). Peak areas that fell below the percentage threshold (ranging from 1 - 3% of the total peak area, depending on the profile) were therefore disregarded.

Sequencing and phylogeny

Clones that contained *amoA* genes which resulted in dissimilar cutting patterns were selected for insert sequencing. Sequences in the GenBank database (NCBI:http://blast.ncbi.nlm.nih.gov) sharing the greatest similarities were selected for multiple alignments using ClustalW2.0.12 software (Larking et al., 2007). Phylogenetic trees were visualised with Treeview software (Page, 1996). Sequences from this study have been deposited in GenBank under accession numbers - .

Results and discussion

Wetland performance

Total monthly flows, hydraulic loading rates (HLR) and nutrient loads were highly variable but followed the climatic pattern of south-west Western Australia, with the highest flows and loads occurring around the rainy winter months (June to October) when large volumes of stormwater runoff carry dissolved nitrogen and phosphorus compounds into the wetland, and the lowest flows and loads occurring during the drier months (November to May) (**Table 1**).

From September 2008 to July 2009 influent TN was predominantly NH_3-N while NO_3-N concentrations were lower. Ammonia removal during this period was highest (> 40% load removed) between December 2008 and May 2009 when HLRs were the lowest (< 7cm/d). The same pattern was observed for TN removal with the exception of March 2009 when no TN load removal occurred. NO_3-N removal varied greatly during the whole period. NO_3-N removal as reported, in terms of influent *vs* effluent loads, does not reflect the total removal capacity of the system as it does not account for the NO_3-N fraction produced by nitrification within the wetland. If we assume that the NH_3-N removed was converted to NO_3-N by nitrification and sum this fraction to the influent NO_3-N then a greater NO_3-N removal can be accepted.

Table 1: Monthly total flows, average concentrations, HLRs, loads and removal rates from September 2008 to October 2009.

DATE	sample	Total Flow	HLR	pH	Concentration (mg/l)				Load applied (g/m2/day)				Load Removal rate							
		(m^3/month)	cm/d		NH_3-N	NO_3-N	TN	TP	NH_3-N	NO_3-N	TN	TP	NH_3-N		NO_3-N		TN		TP	
													(g/m^2/d)	(%)	(g/m^2/d)	(%)	(g/m^2/d)	(%)	(g/m^2/d)	(%)
Sept 08	Inf	55,319	14.4	7.7	93.6	21.1	114.7	15.1	10.53	2.26	12.79	1.69								
	Eff	54,763		7.6	49.8	19.1	69.0	6.3					3.30	31.30	-0.37	-16.29	2.93	22.91	0.81	48.23
Oct-08	Inf	63,100	15.9	7.5	51.7	10.0	61.7	8.2	8.26	1.64	9.90	1.32								
	Eff	62,518		7.4	43.8	8.8	52.5	6.5					1.50	18.11	0.38	23.27	1.88	18.97	0.36	27.63
Nov-08	Inf	33,570	8.7	7.6	26.4	14.4	40.8	7.2	1.71	0.87	2.57	0.35								
	Eff	20,710		7.6	38.8	12.9	51.7	8.9					0.62	36.09	0.54	62.56	1.16	44.98	0.20	56.30
Dec-08	Inf	11,577	2.9	7.7	43.4	22.6	66.0	4.8	1.33	0.67	2.00	0.08								
	Eff	9,835		7.7	18.0	20.9	39.0	5.2					1.15	86.23	0.45	67.51	1.60	79.93	0.02	29.77
Jan-09	Inf	22,865	5.8	7.3	32.8	20.6	53.4	6.3	2.00	1.01	3.01	0.36								
	Eff	20,640		7.5	20.4	13.0	33.3	4.6					0.95	47.67	0.45	44.76	1.41	46.69	0.13	36.59
Feb-09	Inf	20,476	5.7	7.2	37.4	15.6	53.0	6.8	2.14	0.88	3.02	0.21								
	Eff	18,930		7.4	15.7	19.1	34.8	3.5					1.73	80.88	0.33	37.41	2.06	68.19	0.07	35.31
Mar-09	Inf	15,306	3.8	7.4	32.8	13.2	46.0	5.1	1.29	0.47	1.76	0.20								
	Eff	15,490		7.3	19.0	23.3	42.3	5.4					0.56	43.68	-0.57	-121.63	-0.01	-0.54	0.02	8.00
Apr-09	Inf	24,449	6.4	7.5	29.8	17.3	47.1	6.7	1.05	0.66	1.71	0.17								
	Eff	19,742		7.6	20.1	22.6	42.8	4.0					0.65	62.49	0.07	10.67	0.73	42.44	0.07	41.94
May-09	Inf	21,432	5.4	7.7	80.3	33.2	113.5	11.1	3.34	1.83	5.17	0.39								
	Eff	27,601		7.8	45.7	29.4	75.1	7.1					1.88	56.37	0.73	39.96	2.61	50.55	0.21	55.09
Jun-09	Inf			7.5	73.6	33.1	106.7													
	Eff	57,071		7.6	54.3	43.6	98.0	6.6												
Jul-09	Inf			8.6	64.0	25.0	88.9													
	Eff	66,517		7.7	62.7	30.9	93.6	2.3												
Aug-09	Inf	54,767	13.8	8.1	28.0	28.6	56.5		3.55	3.67	7.22									
	Eff	56,323		7.7	20.2	32.6	52.7	2.2					0.73	20.69	-0.69	-18.86	0.04	0.57		
Sep-09	Inf	63,883	16.6	7.5	11.0	32.1	43.4		1.65	4.77	6.42									
	Eff	63,063		7.5	6.0	34.6	40.6	1.8					0.94	56.90	-1.05	-21.99	-0.11	-1.72		
Oct-09	Inf	53,147	13.4	7.9	6.0	38.6	44.8		0.77	4.84	5.62									
	Eff	50,640		7.8	1.0	33.6	34.7	2.2					0.62	80.65	0.70	14.38	1.32	23.53		
Influent [a]	avg	36,521	9.4	7.5	39.8	23.2	63.0	7.1	2.8	2.1	4.9	0.5								
	max	63,883	16.6	8.8	220.0	205.5	405.5	30.1	10.5	4.8	12.8	1.7								
	min	11,577	2.9	6.2	0.03	0.1	1.8	0.0	0.8	0.5	1.7	0.1								
Effluent [b]	avg	38,846		7.6	30.9	24.7	55.6	6.1		**Overall removal rate**[c]			1.2	38.7	0.1	4.2	1.3	25.4	0.2	39.9
	max	66,517		7.8	130.0	71.4	162.1	30.8					3.3	86.2	0.7	67.5	2.9	79.9	0.8	56.3
	min	9,835		7.3	0.0	1.9	10.8	0.0					0.6	18.1	-1.0	-121.6	-0.1	-1.7	0.0	8.0

[a] n=312, [b] n=301, [c] considers total load in whole period (excludes June and July 2009)

This speculative assumption however relies on classical nitrification-denitrification and does not consider other alternative removal pathways such as anaerobic ammonium oxidation (anammox) and complete autotrophic nitrogen removal over nitrite (CANON) which may happen in wetland sediment environments with high ammonia and low oxygen and carbon concentrations as demonstrated by Sun and Austin (2007).

Due to flow meter works, influent flows and loads into wetland cell 1 could not be calculated for June and July 2009, effluent loads in these months however were the highest recorded, indicating that the system was likely overloaded **(Figure 2)**. From August 2009 onwards cell 1 started receiving nitrified effluent from cells 2 and 3, as visible in figure 2 the proportion of NO_3-N increased in the effluent as NH_3-N decreased. There was further removal of ammonia in the wetland cell 1 but nitrate was not removed efficiently resulting in low TN removal in August and September 2009. It is believed that the low COD:N ratio of the wastewater is unfavourable to denitrify the larger nitrate loads. The presence of woodchips in the sand (added at the time of construction, 2004) and the seasonal availability of plant litter are the only organic carbon sources in the wetland.

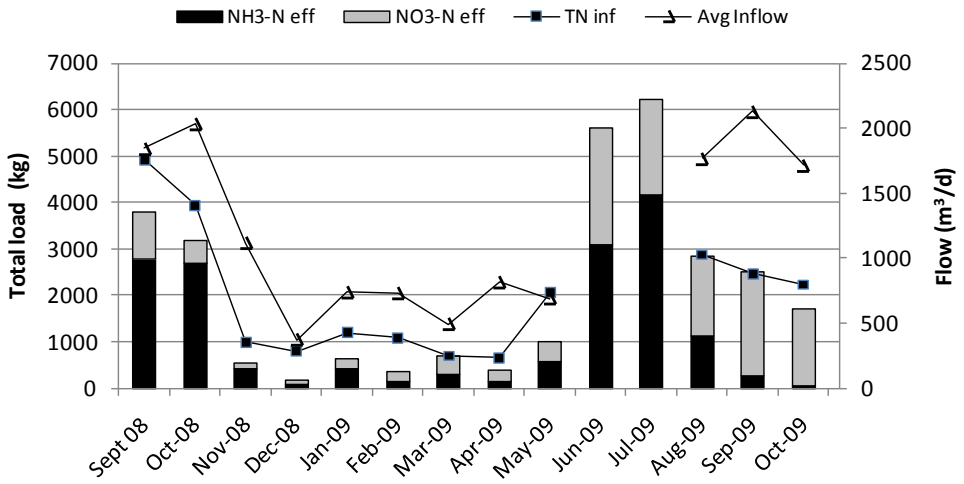

Figure 2: Total monthly loads of influent TN (-■-), effluent NH_3-N and NO_3-N (stacked columns) and average inflow (-Δ-).

The higher percentages of TN removed between November 2008 and May 2009 (except March 2009) are likely to result from a combination of factors such as 1) lower flows and HLRs which mean smaller batches and therefore increased HRT; 2) lower TN loads into the wetland; 3) higher plant litter availability as carbon source for denitrification, as it has been verified the above ground biomass of *Schoenoplectus validus* is mostly dry at the end of winter and forms a thick mat over the surface of the sand during the summer months; 4) higher temperatures (water temperature not recorded, but Perth's average minimum 12.7°C in winter and 24.4°C maximum in summer).

Total load removal for the 12 month period (excluding June and July 2009) was in the order of 39% for NH_3-N, accounting for 5,679kg of NH_3-N removed (removal rate= 1.21g/m^2/d). TN removal was 25% and accounted for 6,067kg of TN removed

(removal rate= 1.30 g/m^2/d). The removal of TP was also significant with 40% of the load or 739kg of phosphorus removed over a 9 month period (removal rate= 0.21g/m^2/day) (see Table 1). The performance of cell 1 in terms of TN removal is similar to other free water surface wetlands treating municipal wastewaters with similar TN influent loads (our study = 1,788g/m^2/yr) and TN influent concentrations >50mg/L (Kadlec and Wallace, 2009). Cell 1 was designed to treat up to 500m^3/day with a targeted 50% TN removal so the 25% TN removal achieved during the period when the flow was on average 1109m^3/day is reasonable considering the system was mostly operated under hydraulic overloads.

AOB analysis by clone library

A total of 80 *amoA* clones were selected from the sludge samples and 56 clones from the sand samples (PCR products from August and September 2008). Sequences were closely associated with *Nitrosomonas nitrosa*, *Nitrosococcus mobilis* (*Nitrosomonas* sp*)* and *Nitrosospira multiformis* (**Figure 3**). Sequences from the sludge and sand were similar with the only exception being a *Nitrosospira*-like sequence present in the sand but not in the sludge. Three unique clone OTUs were most similar to uncultured organisms, clone S1-sand having 99% similarity to anoxic biofilm clone S1 (GenBank:AF202650.1) isolated from a trickling filter biofilm (Schmid et al., 2000) and clones S8-sludge and S9-sand which formed independent OTUs according to HaeIII digestion but were both 99% similar to uncultured bacterium clone AS-AOB-60 (GenBank:GU327814.1) sampled from saline wastewater under low dissolved oxygen. The clone library originated from the sludge samples revealed that 51% of the clones were closely related to *Nitrosomonas* sp. Nm107 (AF272407.1) and 32% related to *Nitrosomonas nitrosa* Nm 90 (AF272404.1). From the sand samples, 84% of clones were closely related to *Nitrosomonas nitrosa* and only 2% associated to *Nitrosospira multiformis* (DQ228454.1). Similarly to our findings, Tietz et al. (2007) verified that the most abundant sequences from VFCWs receiving municipal effluent were related to *Nitrosomonas europaea*/"*Nitrosococcus mobilis*" and *Nitrosospira* sp.

AOB analysis by T-RFLP

The *amoA* based, AluI digest T-RFLP fingerprint revealed four discernible peaks at 224,360, 390, 405 and 491bp. The HaeIII digest revealed 7 discernible peaks at 138, 165, 175, 224, 312, 434 and 491bp and the TaqI fingerprints revealed 3 peaks at 212, 219 and 283bp (**Figure 4**).

There was a higher proportion peaks likely to result from *Nitrosomonas* sp. (AluI-491bp, 405bp and 224bp, HaeIII-165bp and 224bp, TaqI-219bp) than *Nitrosospira* sp (AluI-491bp, HaeIII-175 and TaqI-283bp) in all sludge and sand fingerprints. An exception was the predominance of a *Nitrosospira* sp. peak in the sludge sample from February 2009. Peak AluI-491bp may result from either *Nitrosomonas nitrosa* Nm90 or *Nitrosospira* sp lineages, so the use of HaeIII or TaqI was necessary to differentiate between them. *N. nitrosa* results in a HaeIII-165bp and TaqI-48bp peak while *Nitrosospira* sp results in HaeIII-175bp and TaqI-283bp.

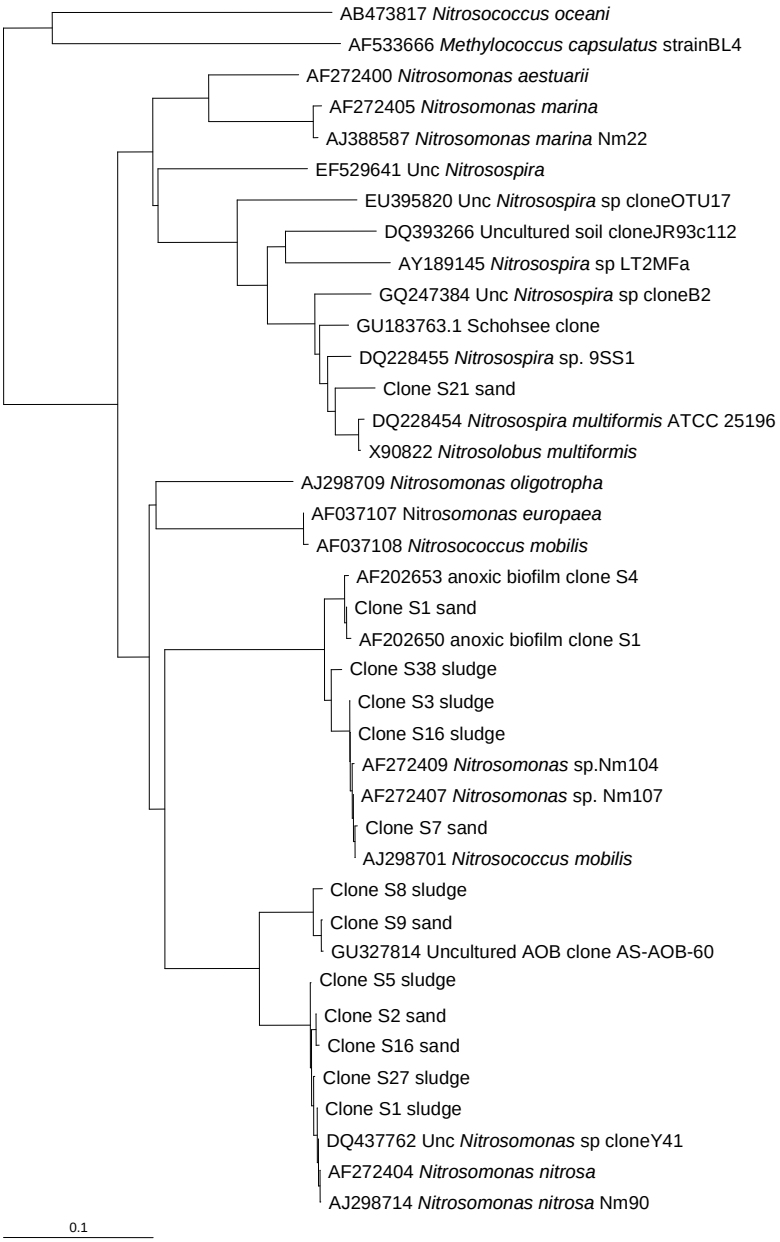

Figure 3: Phylogenetic tree constructed for partial amoA gene sequences of the 13 unique clones obtained in this study and most similar sequences retrieved from GenBank. Neighbour-joining analysis carried out using ClustalW2.0.12. *Methylococcus capsulatus* strainBL4 (AF533666) as outgroup. Scale bar represents 10% nucleotide divergence.

In their TaqI based T-RFLP Horz et al. (2000) verified that all *amoA* sequence types which grouped within the genes *Nitrosospira* showed an OTU of 283bp, indicating the high specificity of the TaqI-283bp OTU to *Nitrosospira* spp. A TaqI-219bp OTU was assigned to members of the *Nitrosomonas* genus, indicative of *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas halophila* and *Nitrosococcus* (*Nitrosomonas*) *mobilis*. *Nitrosomonas nitrosa*–like sequences have a TaqI-48bp peak also indicative of other lineages and was only differentiated by CfoI based T-RFLP which generated a specific 119bp OTU. Even though *N. nitrosa*-like sequences were

common in both sand and sludge samples, the expected TaqI-48bp peak could not be verified in our TaqI based profiles due to noise in peaks smaller than 50bp, its correspondent 165bp and 491bp peaks were common in the HaeIII and AluI profiles, respectively. A TaqI-212bp peak was present in the August and September 2008 samples but no sequence data from clones was retrieved to help assigning its identity.

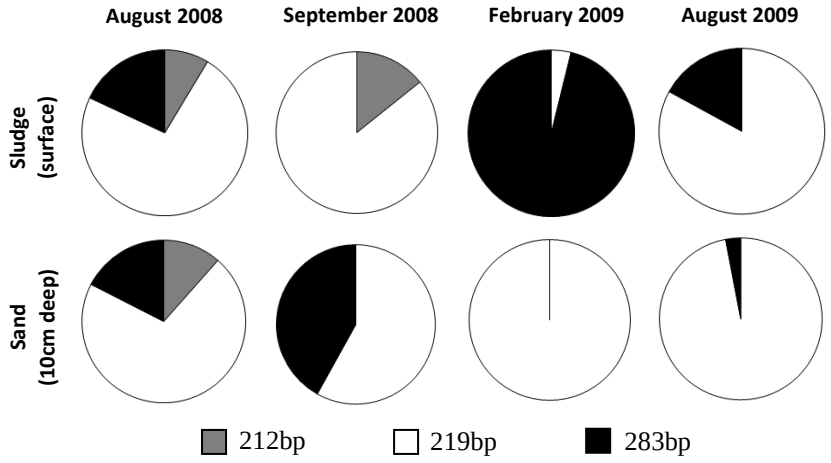

Figure 4: Relative abundance of T-RFs in the CSBP wetland based on TaqI digestion of amoA PCR products. T-RFs: 212bp=(undetermined), 219bp=*Nitrosomonas* sp, 283bp=*Nitrosospira* sp.

Purkhold et al. (2000) verified that Nitrosomonads were responsible for ammonia oxidation in wastewater treatment plants and that *Nitrosospira* were only sporadically present. *Nitrosomonas* spp have a lower substrate affinity but higher maximum activity than *Nitrosospira* spp. therefore *Nitrosospira* spp would be more likely to predominate in low ammonia environments (Schramm et al., 1996) and may be better at withstanding physicochemical variations (Purkhold et al., 2000). However, these findings are just indicative as other studies of systems receiving low ammonia waters (Qin, et al 2007; Regan et al., 2002) revealed that *Nitrosomonas*-like sequences were predominant over *Nitrosospira*-like sequences. Our T-RFLP results support the sequence data. There was a predominance of *Nitrosomonas* sp over *Nitrosospira* sp peaks in the sludge and sand profiles, the *Nitrosospira* sp peak only dominated in the February 2009 sample.

Nitrosomonas nitrosa strains have relatively high affinity constants for ammonia (Koops et al., 2006) and are regarded as having an ecological versatility which allows them to survive in a wide range of substrate conditions (Qin et al., 2007). *Nitrosomonas nitrosa* like sequences have often been isolated from systems with high ammonia concentration such as industrial wastewater treatment plants (Dionisi et al., 2002) and at the same time from low ammonia environments such as freshwater estuaries (Cebron et al., 2003) and a submerged biofilm for drinking water pretreatment in China (Qin et al., 2007). *Nitrosomonas nitrosa*-like sequences were abundant in the wetland cell 1 which receives highly variable NH_3-N concentrations, corroborating the claims of ecological versatility for this group.

Wastewater treatment systems with a high AOB diversity have increased nitrification resistance against perturbation while a monoculture in terms of AOB in a treatment system might render its nitrification efficiency more susceptible (Purkhold et al.,

2000). We have verified that a VFCW receiving inorganic industrial wastewater can sustain a diverse AOB community in its sediments, therefore representing a resilient and robust treatment technology for ammonia removal.

Conclusions

The CSBP wetland cell 1 proved to be efficient in reducing NH_3-N, TN and TP from wastewater prior to its discharge, nitrogen loads and removal performance follow the climatic pattern of south-west Western Australia, with higher loads and lower removal in the rainy winter and lower loads and higher removal in the dry summer. Ammonia removal rates ranged from 0.6 to 3.3g/m^2/day with an overall removal rate of 1.2g/m^2/d. The TN removal rate of 1.3g/ m^2/d is similar to rates reported for free water surface wetlands. The construction of clone libraries in conjunction with T-RFLP revealed a diverse AOB community dominated by *Nitrosomonas* sp members and by less abundant *Nitrosospira* sp. No clear patterns in the composition of the community were observed in relation to depth or time of the year. In August 2009 cell 1 started receiving nitrified effluent from the two new VFCWs, since then its objective has been to achieve denitrification. The addition of more woodchips to cell 1 and dosing of high carbon content wastewaters from nearby industries have been recommended and will be the focus of a future study.

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