NITRIFICATION AND DENITRIFICATION
IN A TIDAL VERTICAL FLOW WETLAND PILOT

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ABSTRACT

This paper describes nitrification and denitrification in a novel tidal vertical flow wastewater pilot treatment wetland (U.S. and international patents pending). The pilot is a flood and drain, serial batch reactor system with process recycle. All wetland cells are covered with mature plants. Tertiary treatment standards ($\leq$ 10 mg/L BOD_5, $\leq$ 10 mg/L TSS, $\leq$ 10 mg/L TN) were achieved from manufactured wastewater (428 mg/L BOD, 48 mg/L TN) without use of aeration machinery. The average hydraulic residence time was approximately 24-hours. Oxygen transfer is a complex process in tidal vertical flow systems. Ammonia ions (NH_4^+) adsorb to media biofilms in the flood stage and then rapidly nitrify when exposed to atmospheric oxygen in the drain stage. Nitrate ions (NO_3^-) desorb into bulk water in the next flood stage and are used as terminal electron acceptors in bacterial respiration. Turnover of the water column during tidal exchanges also promotes atmospheric diffusion of oxygen into bulk water. Apparent simultaneous nitrification and denitrification has been observed directly and indirectly by standard methods, *in situ* probes, and analyses of wetland cell exhaust gases. Genetic probes (fluorescent in situ hybridization - FISH) of biofilm bacteria in this study are consistent with literature results for municipal and industrial wastewater treatment plants except for an elevated percentage of *Planctomycetes*. Results of this pilot study, and information available in the literature, indicate that tidal flow systems can provide advanced, biological nitrogen removal for substantially less energy than activated sludge systems, and occupy a substantially smaller treatment footprint than conventional constructed wetlands for wastewater treatment.

KEYWORDS:

Nitrification, denitrification, constructed wetland, aerobic denitrification, heterotrophic nitrification, vertical flow.

INTRODUCTION

Wetland treatment technology is rapidly evolving. Conventional wetland treatment technologies in common use in the United States are mostly based on passive horizontal flow designs. Vegetated submerged bed (VSB) wetlands can provide reliable secondary treatment performance for BOD_5 ($\leq$ 30 mg/L) and TSS ($\leq$ 30 mg/L), but are ineffective for nitrification (US EPA, 2000). Surface flow wetlands do not consistently meet secondary discharge standards (US EPA, 2000). As stand-alone technology, conventional treatment wetlands are generally inappropriate for applications that must meet strict discharge or reuse standards.

The performance limitations of conventional treatment wetlands are well known within the engineering and regulatory communities. A review of patents and technical literature reveals

recent technological innovations that address these limitations. Key developments are in flood and drain (tidal flow) reciprocating systems (Behrends, 1999), aerated systems (Wallace, 1998), integration of wetlands with nitrifying trickling filters (Reed, 2000), and certain European vertical flow systems (Cooper, 1999) that substantially improve the performance and reliability of wetland treatment technology. These innovations are comprised of mechanical energy inputs, by aeration or hydraulic machinery, that overcome the thermodynamic limitations of passive flow designs that have limited nitrification and, consequently, total nitrogen removal. Additionally, some recent innovations appear to have substantially overcome problems with dispersion and formation of preferential flow channels that have been observed in conventional treatment wetlands. Overall, these recent innovations represent the emergence of a new generation of wetland treatment technologies. The tidal vertical flow technology described in this paper is a member of this set of new technologies.

Tidal vertical flow (TVF) constructed wastewater treatment wetlands operate by cyclic flooding and draining through a media bed with cycle periods of a day or less (Sun et al., 1999). The cycle period of a day or less distinguishes these technologies from other flood and drain systems (Cooper, 1999). Review of scientific literature and data from this study indicate that nitrification and denitrification in TVF wetlands are substantially governed by adsorption chemistry (McBride and Tanner, 2000). In this process, NH_4^+ ions in bulk water adsorb to negatively charged biofilms. Atmospheric oxygen is drawn down into media pore spaces as the TVF wetland drains, causing rapid aeration of biofilms (Behrends, 1999) and subsequent nitrification of adsorbed NH_4^+ ions. Nitrate in biofilms desorbs into bulk water in the next flooding cycle. Nitrate is then consumed as a terminal electron acceptor for bacterial respiration of carbon. The term *tidal nitration*[1] is used here, by analogy to aeration, as a convenient way to denote the transfer of nitrate to bulk water by the ammonia sorption/nitrate desorption process in flood and drain cycles.

Tidal vertical flow is not a new idea in water or wastewater treatment. The first treatment wetland patent taught a type of a tidal flow technology (Monjeau, 1901), but it is not known if any systems were built. Pioneer treatment wetland scientists Seidel (1978) and Wolverton (1983) experimented with flood and drain vertical flow wetlands. Despite early developments, however, tidal flow designs have only recently attracted significant attention from the scientific and engineering communities (McBride and Tanner, 2000. Tanner et al., 1999. Sun et al., 1999. Zoeller and Byers, 1999. Behrends, 1999.). Examples of tidal flow treatment wetlands currently in use are the Village of Minoa, New York (US EPA, 2000) and the Tennessee Valley Authority's reciprocating flow (Recip®) wetlands (Behrends, 1999). The latter technology merits special mention because of outstanding treatment performance and a sound design basis.

Although the idea of tidal flow treatment comes from the end of the 19th Century, scientific knowledge necessary to develop this area of technology comes from the end of the 20th Century. The key scientific insight is the importance of ammonia ion absorption to nitrification in tidal flow systems (McBride and Tanner, 2000. Tanner et al., 1999). Another important insight is the

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[1] The term *nitration* as used in chemistry denotes the attachment or substitution of a nitro group ($-NO_2$) to a molecule.

role of recently identified "novel" bacteria in the nitrogen cycle such as heterotrophic nitrifiers (Robertson and Kuenen, 1990), aerobic denitrifiers (Robertson et al., 1995), and autotrophic denitrifiers (Van de Graaf et al., 1996). Knowledge of both "novel" and classically identified bacteria provides essential insight into the physiological ecology of nitrogen mass transfer within biological systems. Concurrent with the discovery of "novel" bacteria in the nitrogen cycle has been the revolutionary development of genetic techniques to characterize the bacterial community structure of biological treatment systems (Juretschko et al., 2002. Keller et al., 2002). New technology has already emerged as a direct result of recent discoveries and innovations in microbiology (Jetten et al., 2002. Van Loosdrecht and Jetten, 2002. Seyfried et al., 2001).

It has recently become practicable for design engineers to investigate the fundamental biology of wetland treatment systems. Genetic analyses for wastewater bacteria are now commercially available. Treatment wetlands need no longer be treated merely as "black boxes" in the data curve-fitting exercises of input-output analyses. The "black box" approach has yet to produce well-characterized design equations (US EPA, 2000) despite great efforts by a generation of highly qualified researchers. The problem has been insufficient scientific knowledge (Tchobanoglous, 2000), not a lack of engineering or mathematical expertise. Mathematical modeling is a relatively simple task compared to acquiring the necessary scientific insights to advance engineering design. Improved scientific knowledge is the essential prerequisite to significantly advance constructed wetland technology in the 21st Century.

STUDY PURPOSE AND METHODS

The purpose of this study was to explore total nitrogen removal performance in tidal marsh[2] systems for our company's research and development program. We chose a novel configuration (U.S. and international patents pending) of flood and drain marsh cells to achieve a high total nitrogen treatment performance standard. Our study system is comprised of a five-cell TVF marsh pilot treating manufactured wastewater (Table 1, Figure 1, Figure 2), located in Taos, New Mexico at an altitude of 2100 meters. The TVF marsh pilot operates in a serial flood and drain mode using a programmable logic controller (PLC) with pressure transducer input to operate 115-Watt sump pumps that drain and fill marsh cells. Typically, two cells are drained and two are flooded at any one time. A free water surface is established in each flooded cell. Influent is dosed equally into the first three cells (step-feed). There is process recycle in which the volume from the flooded final cell is transferred to the drained first cell. Discharge is by an overflow drain in the final sump. There is no aeration machinery.

The number of flood and drain cycles varied approximately between 3 and 14 cycles per day per cell, with an average of approximately 8 during the period of this study. Variation was not intentional. The program logic used in the PLC had flaws that had to be corrected as discovered.

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[2] The term *marsh* is used instead of wetland for our system in keeping with the technical nomenclature defining a marsh as a wetland with a mineral substrate that does not accumulate peat (Mitsch and Gosselink, 1993).

Light for plants was provided with skylights and 1000-Watt metal halide lamps on reciprocating linear tracks. The PLC turned lights on and off on 12-hour cycles.

Table 1. Tidal marsh pilot design summary.

Influent flow	Mean = 1.7 (σ = 0.5) m^3/d metered in by a four channel irrigation timer into the dosing sump.
Process recycle	3:1 - 14:1 from final to first cell.
Flow regime	Downflow flood and drain
Hydraulic residence time	Average, 24 hours
Manufactured wastewater constituents.	1. Dried cheese whey
	2. 98% urea pearls
	3. Well water
Nominal (calculated) mean influent strength	1. COD: 564 mg/L
	2. BOD: 402 mg/L
	3. TKN: 38 mg/L
	4. NO_3: 3.0 mg/L (well water concentration)
	5. TP: 2.6 mg/L
	6. TSS: < 50 mg/L
Mass dosing schedule	Daily, metered over 24 hours by a mechanical belt feeder.
Total wetland area (five cells)	8.9 m^2 (96 ft^2)
Media depth	0.6 m (2.0 ft)
Media type	9.5mm x 2.4mm expanded shale (Utelite$^{®}$). Slightly modified ASTM C-330
Plant surface coverage	100%

Figure 1. Schematic plan view of TVF marsh pilot. Underdrain system not depicted. Marsh cells have equal volumes.
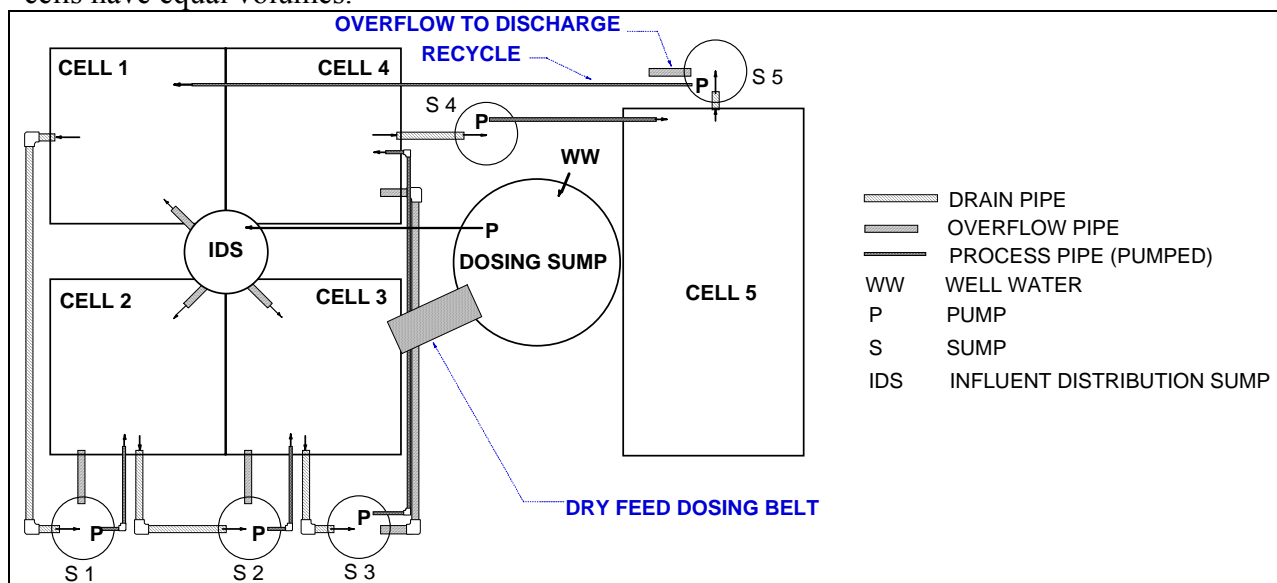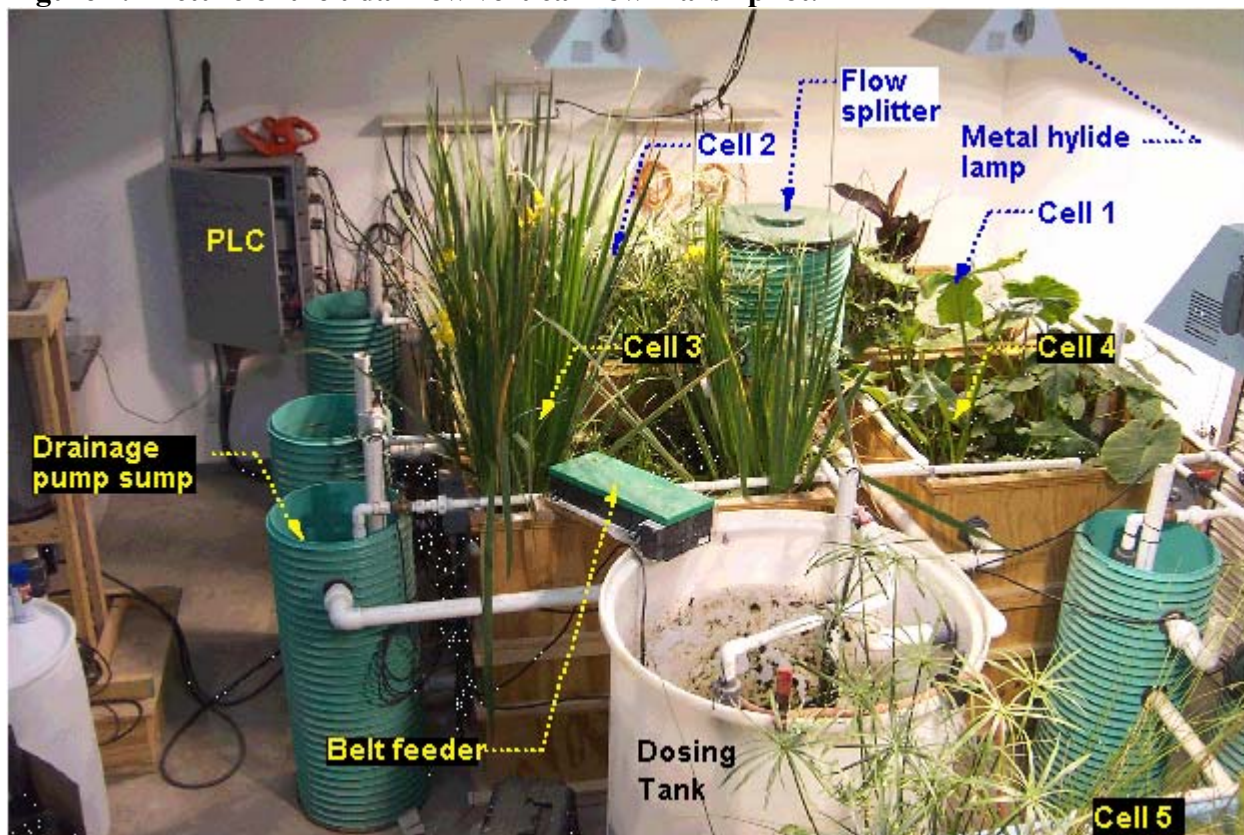
Figure 2. Picture of the tidal flow vertical flow marsh pilot.

Ramp-up of the TVF marsh pilot began in February 2002. Coir mats containing pre-grown wetland plants covered the surface of marsh cells. Marsh cells were inoculated with muck and water from a local pond, and nitrifying biosolids from the clarifier of the local wastewater treatment plant. The pilot was initially configured with four-cells and operated without parallel (step) feed, with the intent to later convert to a step-feed configuration. After six months of operation, a fifth cell was added and the system was converted to step-feed. The conversion was completed in September 2002. This paper presents data gathered on the five-cell configuration during the 4th quarter of 2002. Comparison of step-feed and non step-feed configurations is not covered in this paper.

In our research special attention was given to understanding the performance of and mechanisms for nitrification and denitrification by the following methods:

1. Organic and reduced nitrogen (TKN), nitrate, and nitrite were monitored across the system by standard methods analyses of grab samples from marsh drainage sumps. A certified contract laboratory conducted standard method analyses. BOD and TSS were also monitored.

2. Nitrification and denitrification were directly and indirectly tracked with an *in situ* multi-probe unit (Hydrolab DataSonde®). The probe was programmed to take and log readings every 15-minutes. Parameters measured were ammonia (ISE probe), dissolved oxygen (ISE probe), oxidation-reduction potential (ORP, platinum reference electrode), pH, turbidity

(ntu), temperature, and conductivity. The multi-probe unit was placed in each marsh drainage sump in weekly rotation for a period typically of 24 hours or greater. This method of probe deployment produced a large data set to observe nitrification directly (ammonia ISE probe) and indirectly (DO, ORP, and pH probes) and denitrification indirectly (DO, ORP, and pH probes). All probes were calibrated monthly per manufacturer's instructions.

Turbidity of tertiary effluent at low values (ntu < 10) correlates with low effluent BOD_5 and TSS concentrations. Turbidity values in the pilot can thus provide a strong qualitative indication of the available organic carbon in the recycle. One can infer from low effluent turbidity that there is little organic carbon available in process recycle for heterotrophic denitrification.

Conductivity readings were used to determine submergence of the probe. A sudden, temporary drop in conductivity indicated that the probe was exposed to air. Readings of all parameters from exposed probes were rejected.

3. Exhaust gas samples from wetland reactors were gathered to show evidence of denitrification. Gas samples were gathered from bubbles emerging from the marsh cell underdrain system into drainage sumps. The gas samples were taken from a cell given an extra dose of dried cheese whey[3], to provide excess labile substrate for bacterial metabolism, and 98% urea pearls to provide a high concentration of TKN for nitrification and denitrification. When the cell and adjacent sump were full of the dosed water, the drain pump discharged at a high rate to entrain bubbles downward into the underdrain system that would normally emerge at the surface of the bed. An inverted, submerged, flooded, clear plastic container connected to a 200 ml vacuum evacuated gas collection cylinder was held in the water column over the drain discharge to capture entrained bubbles. When the gas volume trapped in the container displaced water volume in excess of the collection cylinder volume, a valve was opened to fill the collection cylinder with gas. Two gas cylinders were filled. Gas analyses were conducted by a contract lab using three methods: fixed gas analysis for oxygen, nitrogen, hydrogen, carbon monoxide, carbon dioxide, and methane (mol fraction of each gas); ASTM D-5504 for hydrogen sulfide (ppm v / v); and GC-Chemiluminescence for nitric oxide and nitrous oxide (ppm v / v).

4. Biofilm bacteria were characterized by fluorescent in situ hybridization (FISH) genetic probes. Two five hundred milliliter samples of media from the third and fifth marsh cell were combined, fixed in ethanol, and sent to Vermicon AG (Munich, Germany) for testing. At the Vermicon laboratory, 10-gram samples of media were vortexed (3 minutes), then treated in an ultrasonic bath (5 minutes), followed by vortexing (3 minutes). The surrounding liquid was diluted 1:1 with ethanol and mixed. A set of fluorescently labeled gene probes was used to identify specific groups of nitrifying and denitrifying bacteria within the biofilm sample. Quantification for probed bacteria (% of all bacteria) was then conducted with a fluorescence microscope.

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[3] Dried cheese whey is approximately 70% lactose by weight. Lactose is highly labile.

RESULTS

Standard Methods Analyses

Standard methods analyses are presented in Table 2. The effect of recycle can be readily observed in the relatively flat distribution of parameter concentrations across all drainage sumps. The methods used for BOD_5 and TSS analyses had lower detection limits of 4.0 mg/L. Non-detect for BOD_5 and TSS were recorded as 2.0 mg/L. Total nitrogen (TN) values averaged below 10 mg/L with substantial variability in the data due to denitrification. As can be seen in the TKN data, there was little variability in nitrification. The PLC logic structure governing flood and drain protocol was not optimized for denitrification during this study.

Table 2. **Standard methods analyses October-December 2002**.

Parameter, mg/L	Influent	Sump 1	Sump 2	Sump 3	Sump 4	Sump 5, effluent
BOD_5	**428**	**12.0**	**8.2**	**18.4**	**6.8**	**5.2**
n	9	9	9	9	9	9
σ	181.5	22.3	7.9	30.9	7.9	7.0
TN	**48**	**9.6**	**9.2**	**8.2**	**8.1**	**8.3**
n	9	9	9	9	9	9
σ	7.6	7.0	6.8	6.4	6.5	7.2
$NO_3^- - N$	3.0	**6.2**	**5.7**	**5.1**	**6.0**	**7.0**
n		9	9	9	9	9
σ		7.6	5.4	6.3	6.8	7.7
$NO_2^{-2} - N$	Not sampled	**0.05**	**0.30**	**0.35**	**0.27**	**0.13**
n		9	9	9	9	6
σ		0.0	0.6	0.7	0.5	0.20
TKN - N	**45**	**3.4**	**3.5**	**3.2**	**2.0**	**1.3**
n	9	9	9	9	9	9
σ	7.6	3.9	3.0	1.9	1.1	1.2
TSS[4]	< 50 mg/L	**9.8**	**7.1**	**6.1**	**5.8**	**3.5**
n		8	8	8	8	8
σ		12.7	9.1	7.3	6.1	2.1

Hydraulic loading of the TVF marsh pilot was 191 L/m^2 (4.7 gal/ft^2). Mass BOD_5 loading was 81 g BOD_5/m^2 (720 lbs BOD_5/acre). The volumetric nitrification rate, as measured by TKN reduction, was 14.7 g/m^3 media/day (σ = 3.9 $g/m^3/d$), and the areal rate was 8.3 g/m^2/day (σ = 2.2 $g/m^2/d$). Volumetric total nitrogen removal was 13.0 g/m^3 media/day (σ = 2.8 $g/m^3/d$), and

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[4] Removal of total suspended solids could not be comprehensively assessed for this report. The manufactured effluent was highly soluble. Influent TSS was therefore low. Pilot studies are currently underway that use horse manure as a source of TSS.

the areal rate was 7.5 g/m^2/day (σ = 1.6 g/m^2/d). The areal rate is given for comparison to other wetland treatment systems.

Two single-pass experiments were conducted to observe treatment on a single-cell basis. The first experiment observed treatment of a large slug of influent dosed equally to the first three cells. The second experiment observed treatment of a slug of water from one cell as it passed in serial batch mode through the system. In the first experiment, Cells 1, 2, and 3 were drained and then dosed in parallel with approximately 1275 L of water containing 700 grams of dried cheese whey and 200 grams of 98% urea. Influent was sampled prior to dosing. After dosing, the drainage sumps of the respective cells were sampled and analyzed by standard methods for TKN, nitrate, and nitrite. The water level in each sump was measured to confirm that the dosage split was approximately equal. Each cell removed a substantial fraction of total nitrogen (Figure 3). All cells exhibited apparent simultaneous nitrification and denitrification. Hydrolysis of urea, which produces ammonia, and adsorption of ammonia may account for a significant fraction of apparent simultaneous nitrification and denitrification.

Despite the nearly equal 3-way split of the dose, there was a sharp difference in both nitrification and denitrification between the three cells. The reason for the difference is not known, but has a plausible explanation. It is possible that Cells 1 and 2 did not have the same effective media surface area as Cell 3. In an experiment conducted in June-August 2002, Cell 1 was deliberately overloaded with BOD until it exhibited substantial headloss. Cell 2 also exhibited significant headloss. Both cells appeared to hydraulically recover almost completely, but did continue to exhibit dynamic headloss in excess of other cells during the test reported here. When Cells 1, 2,

Figure 3. **Parallel flow nitrification and denitrification from a single influent dose in the TVF pilot.** S1 = Sump 1, etc.
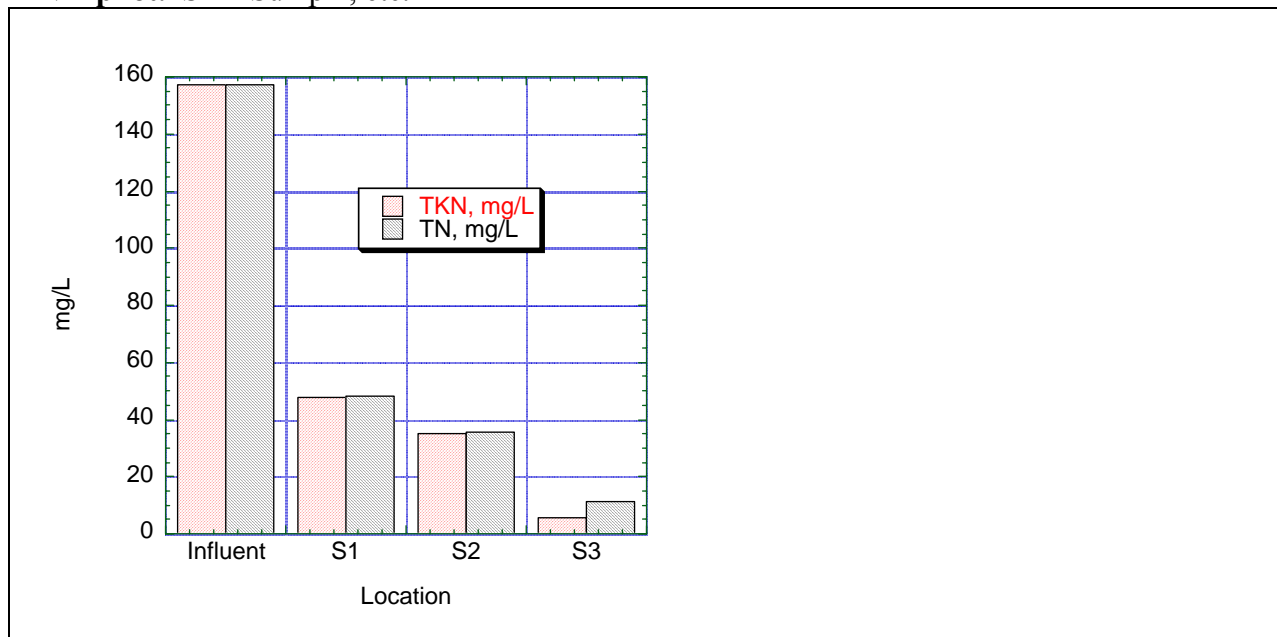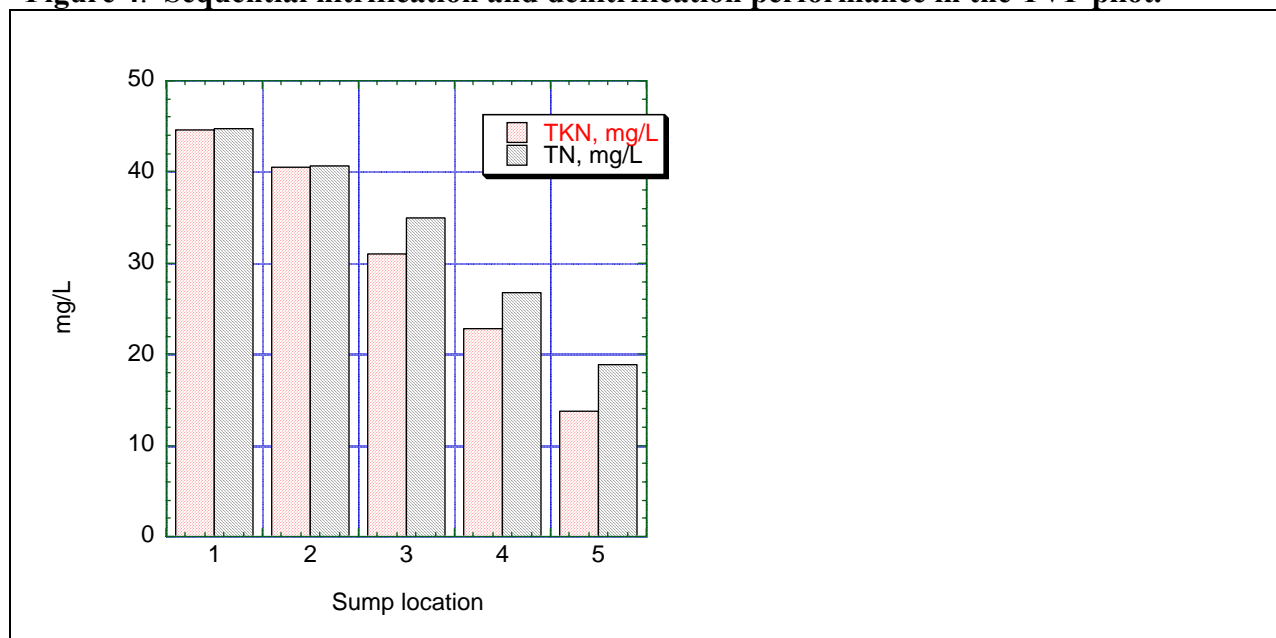
Figure 4. **Sequential nitrification and denitrification performance in the TVF pilot.**

and 3 were excavated in January 2003, media in Cells 1 and 2 appeared to have some clogged zones, Cell 3 did not. These clogged areas would likely reduce the effective surface area of both cells and thus impair their treatment capacity.

The second experiment immediately followed the first. Cells 2, 3, and 4 were drained. The volume in Cell 1 was retained. Then the volume of Cell 5 was recycled to Cell 1 as occurs in normal operation. The drainage sump of Cell 1 was sampled again. The volume of Cell 1 then was pumped successively from cell to cell. After each cell was flooded, a sample was drawn from each sump and analyzed as in the first experiment (Figure 4). Apparent simultaneous nitrification and denitrification occurred in each cell. Average total nitrogen removal per cell was approximately 19% as determined by the difference in TN between each sump after the serial flood and drain cycle was completed.

Multi-probe Analyses

Multi-probe data are consistent with standard method analyses (Table 3, Figure 5). The effects of recycle can be seen in the shallow gradient of ammonia (NH_4^+) and turbidity (ntu) values. The effects of step-feed can be seen in the readings from Sump 3, the final and third cell that receives step-feed. Dissolved oxygen and ORP readings are lowest in Sump 3, which also has the highest BOD_5 values (Table 2). By these results Cell 3 is the most heavily loaded cell. The low turbidity values for Sump 5 are consistent with that sump's low BOD_5 and TSS (Table 2).

Multi-probe effluent ammonia concentration values parallel standard methods analysis of effluent TKN (Figure 5). Influent TKN concentrations are known for all sample days because of the mass loading protocol for manufactured wastewater. Mean nitrification is > 99% as calculated from mean influent TKN and effluent NH_4^+ probe values.

Table 3. Summary of multi-probe samples October to December 2002.

Location & Statistic		D.O., mg/L	ORP, mV	pH	NH_4^+, mg/L	ntu
Sump 1	**mean**	**0.6**	**207**	**8.6**	**2.7**	**9.3**
	n	914	914	914	914	907
	Stdev	0.6	182	0.6	2.5	7.1
	Max	3.3	469	10.3	10.1	53.1
	Min	0.0	-199	7.8	0.3	2.8
Sump 2	**mean**	**0.8**	**208**	**8.4**	**2.1**	**12.9**
	n	512	512	512	512	508
	Stdev	0.8	212	0.7	2.2	12.6
	Max	3.2	425	10.3	8.4	143.8
	Min	0.0	-230	7.6	0.4	3.2
Sump 3	**mean**	**0.4**	**149**	**8.6**	**2.6**	**12.0**
	n	464	464	464	464	450
	Stdev	0.5	183	0.7	1.8	8.7
	Max	3.4	502	10.6	8.5	57.0
	Min	0.0	-215	7.3	0.4	3.2
Sump 4	**mean**	**1.2**	**465**	**7.8**	**2.1**	**7.9**
	n	666	666	666	666	651
	Stdev	0.6	140	0.3	1.5	2.9
	Max	3.6	594	8.6	7.6	25.4
	Min	0.0	-131	6.8	0.5	4.0
Sump 5	**mean**	**1.2**	**479**	**7.9**	**0.9**	**5.6**
	n	1488	1488	1488	1488	1484
	Stdev	0.9	70	0.3	0.6	2.6
	Max	4.2	589	8.4	4.1	51.2
	Min	0.1	334	7.3	0.3	3.3

Oxidation-reduction potential probe analysis reveals indirect evidence of nitrification and denitrification. Dissolved oxygen concentrations and ORP values are partially decoupled in the TVF marsh pilot. Desorption of nitrate ions into bulk water in the flooded state can be seen in data sets (collected on separate days under the same dosing protocol) in which the ORP values cycle while DO concentrations remain unchanged or change only slightly (Figure 6, Figure 7, Figure 8). Dissolved oxygen is not consumed by nitrification because nitrification takes place in biofilms exposed to the atmosphere. Nitrification therefore positively influences ORP. Although a significant mass of oxygen is transferred to bulk water by atmospheric diffusion during drain and flood cycles (see Discussion), the ORP and BOD_5 analyses provide strong evidence that nitrate drives a substantial part of the oxidation of organic carbon.

Figure 5. Nitrogen treatment frequency distribution summary comparing standard method analyses (TN, TKN) with multi-probe analysis (NH$_4^+$). Sample size for NH$_4^+$ is n = 1484.
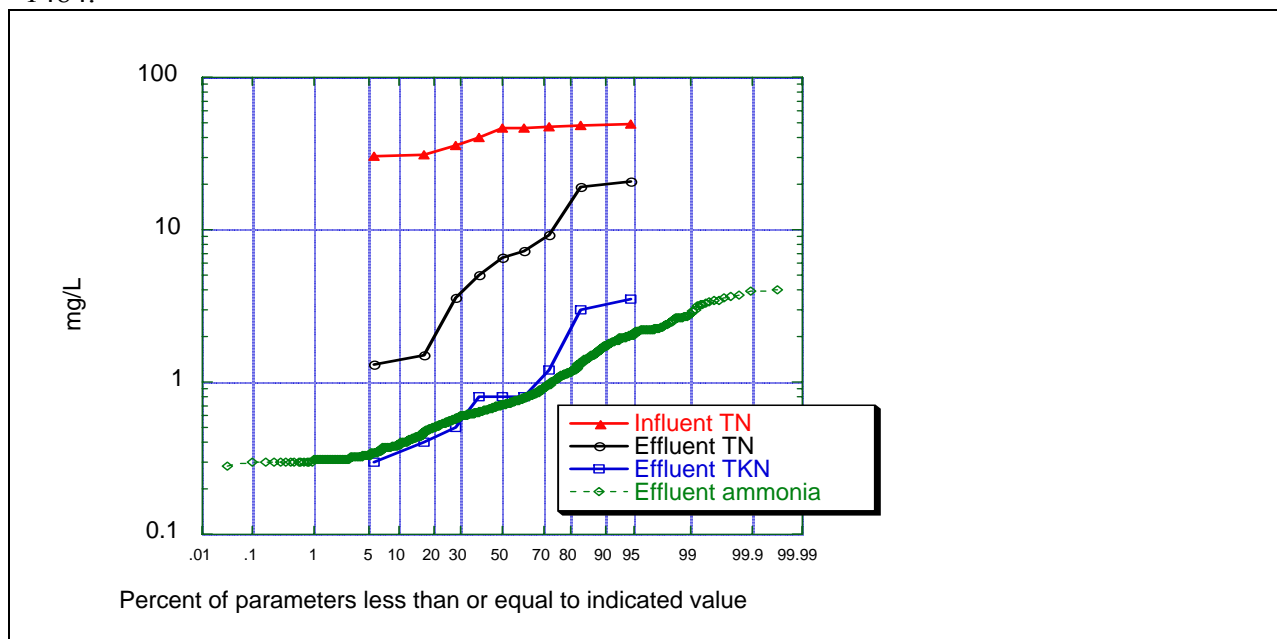

Figure 6. Simultaneous depression of DO and increase of ORP in Cell 5 drainage sump. The effect of the doses on Cell 5 lags until flood and drain cycles carry the mass load forward.
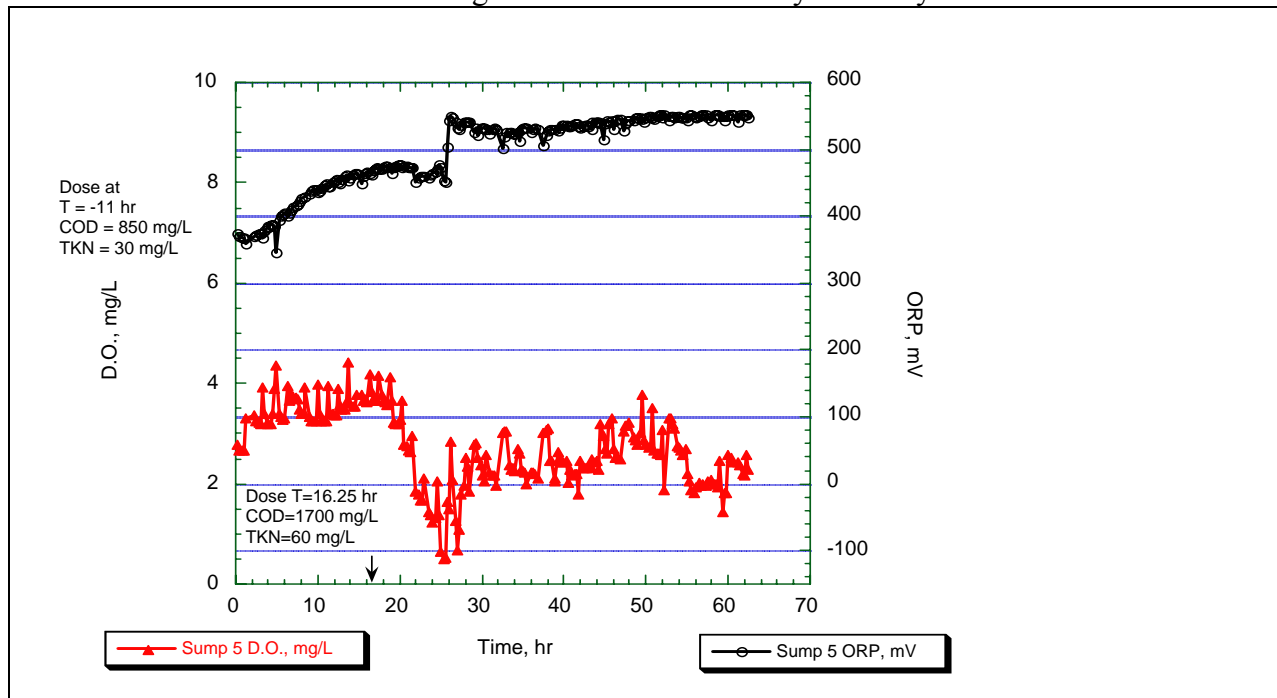
Figure 7. Cycling of ORP in Cell 3 drainage sump from nitrate influx in flood and drain cycling. Influx of TKN is from activation of the irrigation timer feeding the dosing sump.
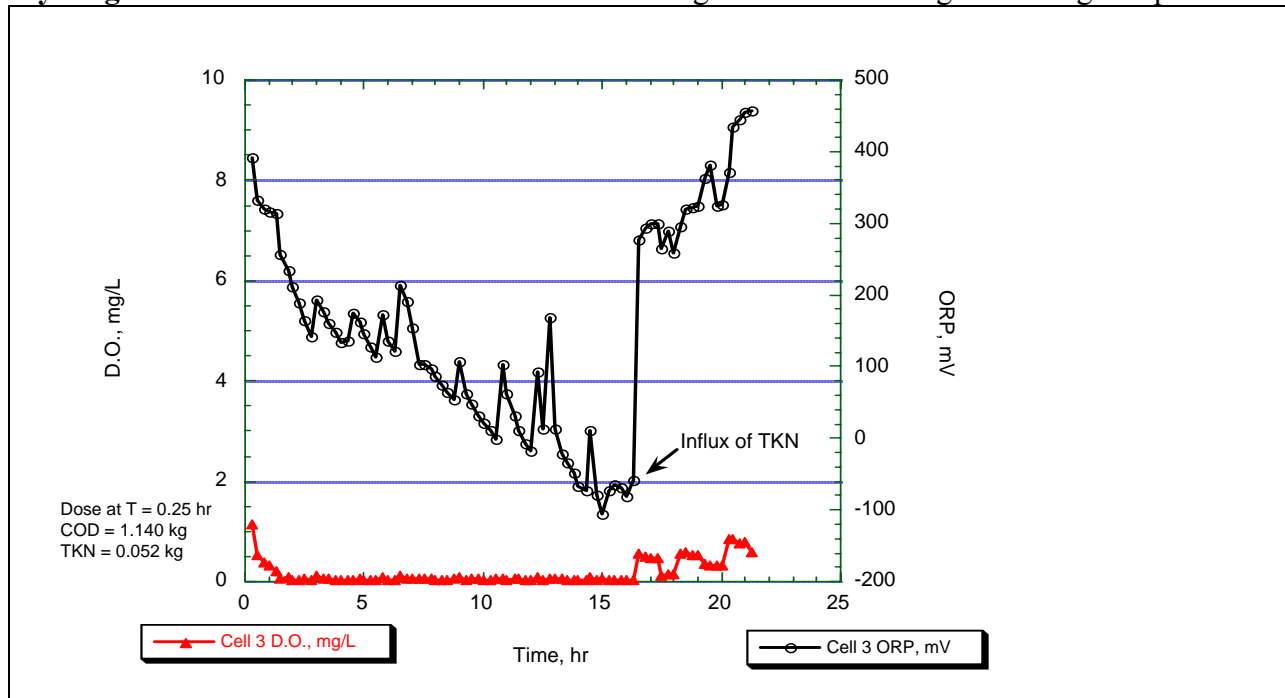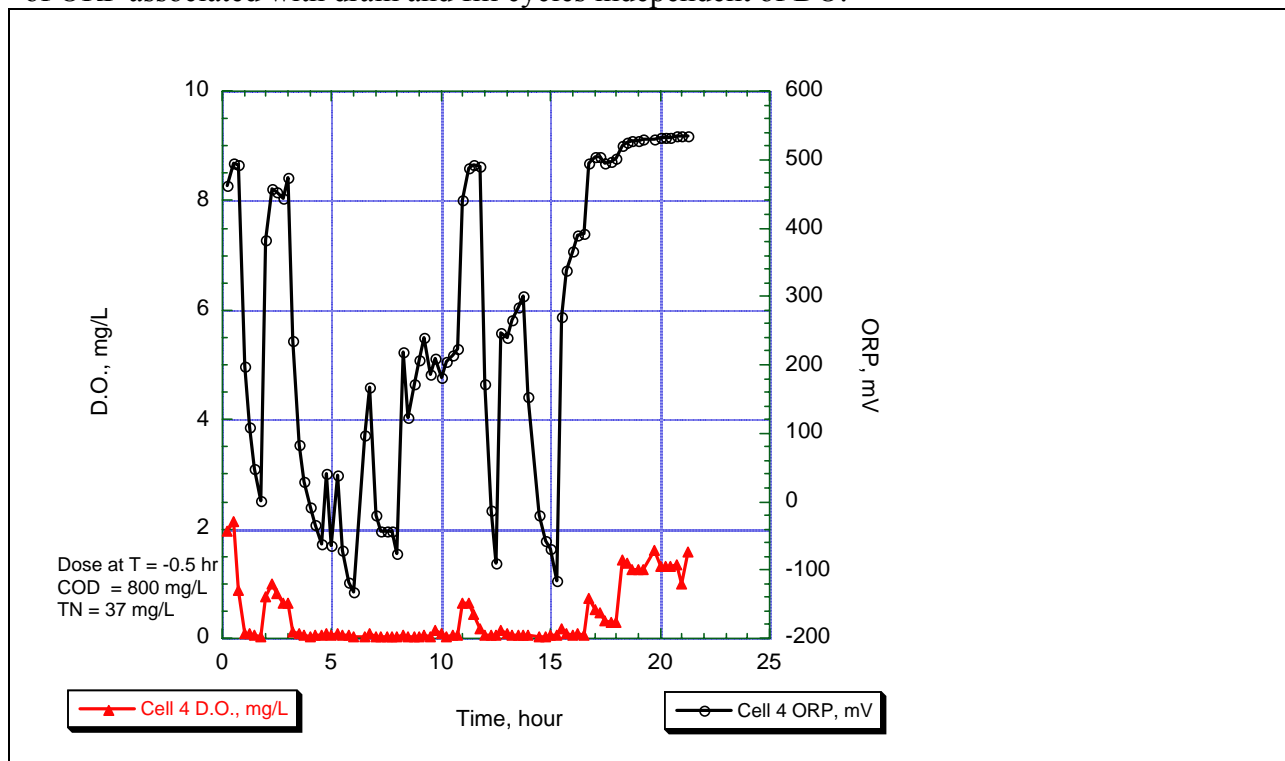

Figure 8. Concurrent high ORP and low DO values in Cell 4 drainage. Note cyclic peaking of ORP associated with drain and fill cycles independent of DO.

Gas Analyses

Analysis of exhaust gas from the marsh cells provides additional insight into denitrification within the TFVM marsh pilot (Table 4). Marsh cells receiving the most organic carbon loading were observed to bubble, sometimes vigorously, uniformly across the bed for periods of up to a half hour. Air trapped in underdrain piping and the media seemed an unlikely source for large volumes of prolonged, uniform bubble production. Sampling gas directly from the media beds to get the required 200 ml sample volume was impracticable. By fully opening the pump discharge valve, bubbles could be entrained by downward flowing water in flooded sumps. Large bubbles emerging from the drainpipe into the flooded drain sump were easy to capture.

After obtaining the first gas sample from Sump 1, we realized that the air in the drainpipe had not been fully purged. Repeated fill and drain cycling of Sump 1, while keeping the drainpipe discharge submerged, appeared to completely purge the drainpipe of trapped air. A second gas sample was then obtained from Cell 1 after it was flooded with influent dosed with dried cheese whey and urea.

Evidence for denitrification can be found in both samples in the increase of atmospheric nitrogen (N_2) mol fraction and nitric oxide (NO) ppm concentration (Table 4). Both gases are end products of denitrification (summarized in Barton and Atwater, 2002). Nitric oxide may also be produced in nitrification (Jetten et al., 2002). Sample 2, which was purged of air trapped in the drainpipe, exhibits advanced denitrification as can be seen in the high nitrogen, high carbon dioxide, and low oxygen mol fractions. The percent difference between the NO concentrations in the samples and the reference atmosphere is semi-quantitative. Nitric oxide is a highly reactive gas without a stable mol fraction in the atmosphere, making impossible a precise percent difference calculation between the reference atmosphere and the gas sample.

Table 4. **Marsh sump exhaust gas analysis.** The reference atmosphere is calculated at 2100 meters.

Analytical method	Gas	Reference atmosphere	Sample 1	Sample 2	Unit	Sample 1 ∂ Atm, %	Sample 2 ∂ Atm, %
Fixed Gas Analysis	O_2	20.9	19.4	5.2	Mol %	-7.4	-75.3
	N_2	78.1	79.6	90.4	Mol %	1.9	15.8
	H_2	0.00050	< 0.10	< 0.10	Mol %		
	CO	trace	< 0.01	< 0.01	Mol %		
	CO_2	0.00330	0.39	3.67	Mol %	11,700	111,100
	CH_4	0.00007	0.62	0.72	Mol %	947,000	1,100,000
ASTM D-5504	H_2S	trace	< 1	< 1	ppm v / v		
GC-Chemiluminescence	NO	low ppb	4	4	ppm v / v	~100,000	~100,000
	N_2O	0.5	< 1	< 1	ppm v / v		

The gas samples also reveal methane production and denitrification in the presence of oxygen. Aerobic denitrification is well known (Robertson et al 1995), but methane production is less easy to explain. Anaerobic micro-sites within the media bed may be a source of methane production. However, Behrends (1999) reports that oxygen saturation of media biofilms in a tidal system takes place within one minute of atmospheric exposure. Oxygen saturated biofilms would likely inhibit methanogenesis, but it is possible that methanogenesis may not be immediately suppressed by high oxygen concentrations. Kato et al. (1997) report high oxygen tolerance of methanogenic bacteria located on granular, activated sludges. Thus the presence of anaerobic micro-sites is not a facile, complete explanation of methane production in a complex biofilm environment.

The analytical method used for hydrogen sulfide (H_2S) was insensitive to concentrations of less than 1 ppm. We did notice an H_2S odor in the gas test. The human odor threshold for H_2S is 8.1 ppb (Amoore and Hautala 1983). A more sensitive method of analysis is required for quantitative analysis of H_2S exhaust gas.

Microbiology

The bacterial community was characterized by FISH probes in two rounds of testing (Table 5, Figure 9, Figure 10). The first round was used to decide which follow-up tests would be useful[5]. Approximately 70% of all bacteria in the treatment system were characterized by FISH analysis to division, 45% percent of bacteria were identified to genus or group (e.g. *Pseudomonas* sp. or *Azoarcus* / *Thauera* group), and 30% percent of bacteria were identified to species, or species and related bacteria (e.g. *Paracoccus denitrificans* or *Zooglea ramigera* and related bacteria). The composition of the bacterial community in the TVF pilot appears to be similar to activated sludge systems (Beimfohr, 2003. Juretschko et al., 2002).

Given the physiological (functional) diversity between species within the same genus, the bacterial community in the TVF marsh pilot is probably not well characterized. For instance, *Alcaligenes faecalis* is a heterotrophic nitrifier / aerobic denitrifier (Inamori et al., 1997), but *Alcaligenes latus* does not have these physiological characteristics. Although it may be tempting to infer physiological functions across species within a genus or group, yielding to this temptation is likely to prove embarrassing. The 70% of the bacterial community that is not characterized by species leaves substantial room for physiological diversity.

When the bacterial community is categorized by function (Figure 10), approximately 41% of known species, genera, or groups in the TVF pilot play a role in nitrogen mass transfer. Not included in the 41% are *Pseudomonas* sp. (4%), a genus with many denitrifiers, and *Planctomycetes* (14%). Some *Planctomycetes* are autotrophic denitrifiers, such as Anammox bacteria (Van der Graaf, 1996). Anammox bacteria were found in the TVF marsh pilot in small numbers, < 1 % of all bacteria. Other bacteria that play a significant role in nitrogen mass transfer may not have been identified. Stevens et al. (2002) identified many Gram-positive,

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[5] The assistance of Dr. Claudia Beimfohr of Vermicon AG was invaluable in the selection of FISH probes.

nitrifying heterotrophic bacteria, of which *Staphylococcus*, *Micrococcus*, *Streptococcus*, *Pseudomonas*, and *Bacillus* species were prominent. Of these genera, only *Pseudomonas* was probed in this study, but not at a species level.

Table 5. Bacterial community structure of TVF marsh pilot by quantitative FISH analysis.

Analyzed Target Division	% of all bacteria	Analyzed target organisms	% of all bacteria	Features
Alpha-subclass of ***Proteobacteria.***	**11%**	*Paracoccus denitrificans*	9.0%	Aerobic denitrifier, heterotrophic nitrifier
		Hyphomicrobium vulgare	< 1%	Facultative denitrifier
		Nitrobacter	< 0.1%	Nitrite oxidizer
		Other Alpha-*Proteobacteria*	2.0%	
Beta-subclass of ***Proteobacteria***	**28%**	*Nitrosospira (Nitrosospira tenuis*-lineage)	5.0%	Ammonia oxidizer
		Nitrosomonas marina-lineage	3.0%	Ammonia oxidizer
		Nitrosomonas oligotropha-lineage	2.0%	Ammonia oxidizer
		Nitrosomonas communis-lineage	< 1%	Ammonia oxidizer
		Nitrosomonas cryotolerans-lineage	< 0.1%	Ammonia oxidizer
		Nitrosomonas eutropha, N. europea	< 1%	Ammonia oxidizer
		Nitrosococcus mobilis	1.0%	Ammonia oxidizer
		Alcaligenes faecalis	< 0.1%	Aerobic denitrifier, heterotrophic nitrifier
		Alcaligenes latus and related bacteria	2.0%	Facultative denitrifier
		Brachymonas denitrificans and related bacteria	< 0.1%	Facultative denitrifier
		Zooglea ramigera and related bacteria	8.0%	Facultative denitrifier
		Azoarcus / Thauera group	7.0%	Facultative denitrifier
		Other Beta-*Proteobacteria*	< 0.1%	
Gamma-subclass of ***Proteobacteria***	**12%**	*Pseudomonas* species	4.0%	Most true *Pseudomonadacae.* Denitrifiers.
		Other Gamma-*Proteobacteria*	8.0%	
Group of Green Non-Sulfur Bacteria	**2%**	**Group of Green Non-Sulfur Bacteria**	**2.0%**	Filamentous. Diverse ecology and physiology.
Group of ***Planctomycetes***	**13%**	Anammox bacteria	< 1%	Autotrophic denitrifiers
		Other *Planctomycetes*	13.0%	Diverse ecology and physiology
Nitrospira	**4%**	***Nitrospira***	**4%**	Nitrite oxidizer
Holophagal / Acidobacteria	< 0.1%	***Holophagal / Acidobacteria***	< 0.1%	
Other bacteria	**30%**	**Other bacteria**	**30%**	

Figure 9. Bacterial community structure of TVF marsh pilot by quantitative FISH analysis. Population < 1% assigned 0.1% value, < 0.1% not listed.
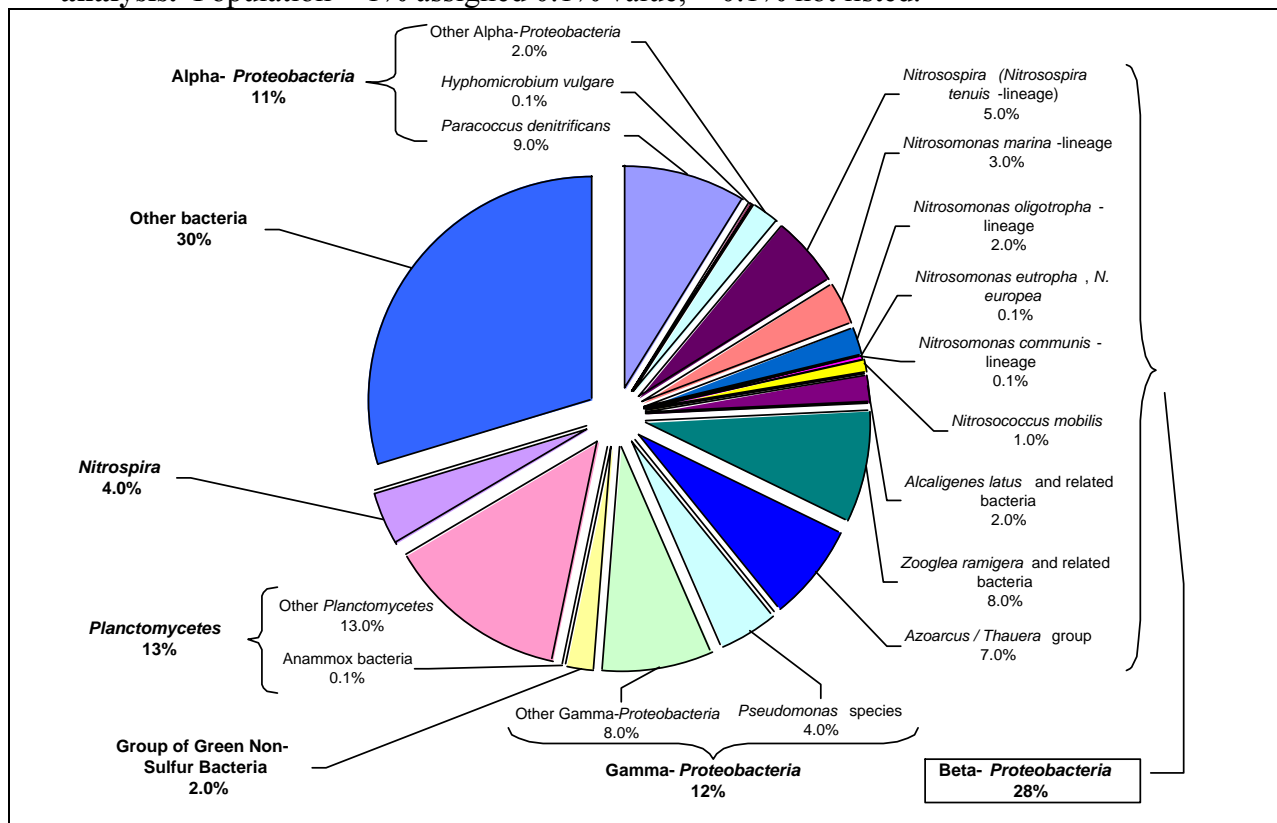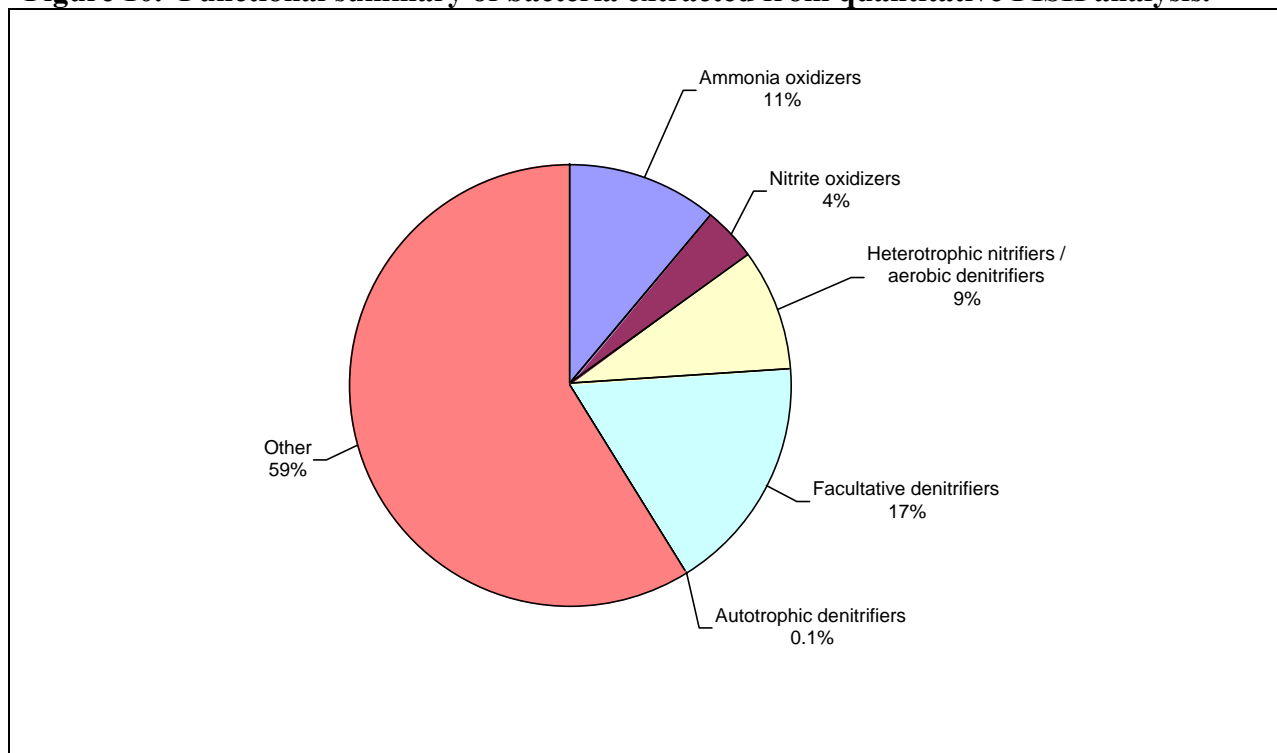

Figure 10. Functional summary of bacteria extracted from quantitative FISH analysis.

It should be noted here that FISH probes exist for known bacteria only. Even so, many known bacteria do not yet have FISH probes available. Thus FISH analysis may miss species (or higher taxonomic classifications) vital to ecosystem function. A comprehensive characterization of a microbial community requires other complementary techniques such as full-cycle rRNA analysis (Juretschko et al 2002) to identify most known bacteria and observe the presence of unknown or partially described bacteria.

DISCUSSION

The TVF marsh pilot appears to be able to process a substantially higher BOD_5 mass loading than vegetated submerged beds (VSB) reported by the EPA (2000). The mean mass loadings of 81 grams $BOD_5/m^2/d$ (720 lbs BOD_5/acre/d) is approximately 13 times higher than the mass loading recommended by the EPA for VSB systems. Similar loading rates have been reported for other tidal flow systems (Behrends et al., 2000). It is wrong to conclude, however, that tidal flow systems should be sized 13 times smaller than standard VSB designs. More conservative sizing criteria are undoubtedly appropriate.

The high loading rate of this study did not cause the pilot to clog, but did result in several inches of dynamic headloss in excess of clean media. Thermodynamically, a tidal flow wetland should be able to treat much higher mass loading than a VSB because of the repeated exposure of biofilms to atmospheric oxygen (~275 mg O_2/L). When exposed to the atmosphere endogenous respiration in biofilms is rate limited by substrate, not oxygen. Long-term studies are currently underway to assess endogenous respiration rates of marsh cell biofilms and accumulation of biomass. Insight into the mechanisms and biology of endogenous respiration rates will be key to a rational design basis for mass loading. Maximum design mass loading of BOD is likely to be the master design constraint for sizing a tidal flow system for total nitrogen removal.

Total nitrogen removal in the TVF marsh pilot is sensitive to the rate of tidal cycles. Sensitivity results from denitrification, not nitrification (Figure 5). Nitrification is less sensitive to tidal cycling rate than denitrification. Studies are currently underway to optimize denitrification. A total nitrogen effluent concentration of less than 3.0 mg/L appears feasible given the right cycling protocol.

Energy efficiency is a key feature of the technology described in this paper. Tidal vertical flow systems rely substantially on *tidal nitration* to drive consumption of organic carbon in wastewater. Tidal fluxes also transfer oxygen directly into bulk wastewater by atmospheric diffusion across water surfaces.

Oxygen transfer efficiency of mechanical aeration processes (kW hr / kg O_2 transferred) is limited by the low solubility of oxygen in water. Clean, cold freshwater saturates at 10 to 12 mg/L O_2. Saturation of O_2 in wastewater is substantially less. The actual (field) oxygen transfer efficiency of mechanical aeration processes in wastewater is typically on the order of 4 - 16%, depending on a variety of factors (Crites and Tchobanoglous 1998).

In contrast to oxygen, nitrate is highly soluble in water. Organic nitrogen and ammonia (TKN) is typically present in wastewater in concentrations from 20 to 85 mg/L (Crites and Tchobanoglous 1998), which is several times that of the saturation value of oxygen in wastewater. Low-head, high-volume pumps used in draining marsh cells have mechanical hydraulic transfer efficiencies on the order of 80 - 90%. Therefore in tidal flow systems TKN is converted to nitrate via a mechanical means that is more efficient than mechanical transfer of oxygen to bulk wastewater. On a mass basis nitrate has the same oxidizing capacity of 2.86 kilograms of oxygen (US EPA 1993). Consequently, the energy efficiency for BOD (or COD) removal of the *tidal nitration* process is greater than aeration of bulk water.

Insight into the energy efficiency of *tidal nitration* and oxygen transfer in TVFM systems is straightforward using classic oxygen demand stoichiometry and oxygen transfer.

Equation 1. Bulk water oxygen demand for activated sludge processes[6]

$$\text{mg/L } O_2 \text{ demand} = (COD_{influent} - COD_{effluent}) + 4.6(NO_3)_f - 2.86(NO_3)_u$$

Where: COD = chemical oxygen demand, mg/L
          $(NO_3)_f$ = nitrate formed, mg/L
          $(NO_3)_u$ = amount of nitrate utilized, mg/L.

The nitrification oxygen demand term $(4.6(NO_3)_f)$ for bulk water oxygen demand drops out in the TVF marsh process because nitrate is formed in drained marsh cells. The revised oxygen demand for bulk water is as follows:

Equation 2. Bulk water oxygen demand for the TVF marsh pilot.

$$\text{mg/L } O_2 \text{ demand} = (COD_{influent} - COD_{effluent}) - 2.86(NO_3)_u$$

In Equation 2 a significant fraction of COD is consumed by nitrate formed in flooded marsh cells. The difference in oxygen demand as calculated by the above equations for the same wastewater flow and influent parameters is 41% (Table 6). The reduction in bulk water oxygen demand by *tidal nitration* is clearly sensitive to the influent COD:TKN ratio.

Does the pumping process required for *tidal nitration* meet the remaining bulk water oxygen demand? As marsh cells flood and drain, marsh cell water volumes undergo repeated turnover throughout the day. This turnover process facilitates transfer of oxygen by diffusion from the atmosphere.

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[6] The sludge wasting term is left out because wasting is highly influenced by specific system characteristics.

Table 6. Comparison of bulk-water oxygen demand.

System	Influent COD, mg/L	Effluent COD, mg/L	Influent TKN, mg/L	% nitrification	Nitrate formed, mg/L	Effluent nitrate, mg/L	Bulk water oxygen demand, mg/L
Activated sludge (MLE)	400	10	45	95%	42.75	5.0	479
TVF Marsh	400	10	45	95%	42.75	5.0	282
Equation 1	mg/L O_2 = (400-10) mg/L + 4.6(42.75) mg/L – 2.86(42.75-5.0) mg/L						
Equation 2	mg/L O_2 = (400-10) mg/L – 2.86(42.75-5.0) mg/L						

Equation 3. **Oxygen transfer (OT) from the atmosphere across TVF marsh pilot surfaces.**

$$OT = (K_d \times C_T \times 24 \text{ hr/d} \times V \times 1000 \text{ L/m}^3 \times 10^{-3} \text{ g/mg}) / \text{area}$$

Where: K_d = oxygen transfer coefficient, hr^{-1}
C_T = oxygen saturation value at temperature T, mg/L
V = bulk water volume, m^3
Area, m^2

The oxygen transfer coefficient is empirically derived and is a function of the dissolved oxygen concentration, temperature, and turbulence of the bulk water. A value of $K_d = 0.10$ is used for wastewater volumes with temperatures at 15°C that are turned over throughout the day. Oxygen saturation in wastewater at 15°C is approximately 9.00 mg/L. Values from a pilot study of volume = 3.2 m^3 and area = 9.4 m^2 are used to complete a calculation of Equation 3.

Equation 4. **TVF marsh bulk water specific oxygen transfer calculation.**

$$OT = (0.1/\text{hr} \times 9.00 \text{mg/L} \times 24 \text{ hr/d} \times 3.2 \text{ m}^3 \times 1000 \text{ L/m}^3 \times 10^{-3} \text{ g/mg}) / 9.4 \text{ m}^2 = 7.5 \text{ g } O_2/\text{m}^2/\text{d}$$

Turnover of the bulk water volumes increases the effective daily transfer surface area. Daily flood and drain cycles increase the effective oxygen transfer area by a factor proportional to recycle flow, taken here for simplicity to be eight.

Equation 5. Daily oxygen mass transfer to the TVF marsh pilot.

$$8 \times 9.4 \text{ m}^2 \times 7.5 \text{ g } O_2/\text{m}^2/\text{d} = 540 \text{ g } O_2/\text{d}$$

Is 540 g O_2/d sufficient oxygen transfer to meet the required oxygen demand of bulk water in the pilot?

Equation 6. **Daily oxygen mass requirement of bulk water in TFV pilot.**

282 mg/L x 1.7 m^3/d x 1000 L/m^3 x g/1000mg = 479 g O$_2$/d

Equation 5 and Equation 6 are essentially in agreement with observations of the pilot (Table 2, Table 3). Both empirical observation and simple stoichiometry confirm that tidal flood and drain cycles promote sufficient transfer of atmospheric oxygen to satisfy oxygen demand unmet by nitrate. Experiments are in progress to determine TVF marsh oxygen transfer kinetics with more precision.

From the beginning of this study, the affect of *tidal nitration* on the composition and physiological ecology of the bacteria community was of great interest. Despite the apparent need to make the characterization more comprehensive, we tentatively conclude that the TVF marsh pilot bacterial community is similar to that of activated sludge. The similarity is not surprising because part of the pilot ramp-up process included a dose of nitrifying activated sludge. Yet nitrification and denitrification within the marsh cell reactors does not behave like activated sludge, even for reactors in series in a Modified Ludzack-Ettinger process (Austin et al., 2000).

At this time, there appears to be no justification to postulate the existence of novel bacteria as responsible for simultaneous nitrification and denitrification in the TVF pilot. Rather, it seems more plausible that the familiar cast of bacterial players in activated sludge can expand their repertoire when acting on a novel stage.

The *tidal nitration* process practically eliminates BOD inhibition of nitrification because nitrification takes place when cells are drained. A high rate of nitrification takes place in all parts of the TVF marsh pilot. On the other hand, there is sufficient available organic carbon substrate (BOD) for denitrification when cells are flooded. Inhibition of nitrification by high BOD:TKN ratios is, in contrast, a standard feature of activated sludge treatment, limiting nitrification to locations (in time or space) where BOD is substantially consumed. Substrate limitation of denitrification is also common in activated sludge systems.

Exposure to atmospheric oxygen concentrations (~275 mg/L) does not appear to substantially inhibit denitrification in the TVF marsh pilot. FISH analysis and a review of the physiological characteristics of known bacteria provide insight into biological foundations of denitrification in the pilot. Nine percent of all pilot bacteria identified by FISH analysis are *Paracoccus denitrificans*, an aerobic denitrifier / heterotrophic nitrifier. *Paracoccus denitrificans*[7] is a constitutive denitrifier that uses both nitrate and oxygen as terminal electron acceptors without a time delay for mobilization of nitrate reductase enzymes (Robertson and Kuenen 1990). Until recently regarded as novel, it became commonly known among microbiologists by the mid 1990's that a wide variety of bacteria have this physiological characteristic (Jetten et al., 2002. Keller et al., 2002. Stevens et al., 2002. Helmer and Kunst, 1998. Robertson et al., 1995. Robertson and Kuenen, 1990. Lloyd et al., 1987). The presence of *P. denitrificans* partially

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[7] *Thiosphaera pantotropha*, subject of many references in this paper, has been reclassified as *Paracoccus denitrificans*.

explains total nitrogen removal in the TVF marsh pilot. The presence of other heterotrophic nitrifiers cannot be ruled out with the available data.

Facultative denitrifiers, in contrast, are classically described as requiring a delay period of 4 to 8 hours to mobilize nitrate reductase (Robertson and Kuenen, 1990). Facultative denitrifiers, by definition, use either oxygen or nitrate as a terminal electron acceptor, but not both at the same time. Bioenergetic considerations favor facultative over constitutive denitrification because producing one set of enzymes, rather than two at the same time, is a substantially smaller metabolic cost. On the other hand, ecological conditions can tilt the advantage to constitutive denitrifiers. Constitutive (aerobic) denitrifiers will have an ecological advantage over facultative denitrifiers in environments subject to frequent, sudden, alternating onset of anaerobic and aerobic conditions (Robertson and Kuenen, 1992. Robertson and Kuenen, 1990. Robertson and Kuenen, 1984). Tidal flow produces exactly those ecological conditions. The range and frequency of oxygen concentrations and ORP values that biofilms are subjected to in tidal flow systems cannot be produced in either activated sludge or conventional wetland treatment systems.

Conditions favoring constitutive denitrifiers do not exclude facultative denitrifiers. Genetic probes (FISH) from this study unequivocally demonstrate that both constitutive and facultative denitrifiers thrive in the same volume, as they do in activated sludge systems (Juretschko et al., 2002). Facultative denitrifiers differ widely in their tolerance to oxygen concentrations before production of nitrate reductase is suppressed (Robertson and Kuenen, 1990). Some of the facultative denitrifiers identified by FISH probes in this study have substantial tolerance to the presence of oxygen. These organisms are *Zooglea ramigera*, *Pseudomonas* sp., and *Alcaligenes* sp. (Robertson and Kuenen, 1990), which were 8%, 4%, and 2%, respectively, of all bacteria. Their denitrification activity is unlikely to be significantly suppressed by periodic exposure to high oxygen concentrations.

The complex environment of a tidal marsh system may complicate classic concepts of enzyme mobilization for facultative denitrifiers. Experiments done to determine rates of enzyme mobilization in wastewater treatment systems are conducted in well-mixed reactors. Media beds in a tidal marsh system are not well mixed. Richaume et al. (2002) report that, in contrast to pure liquid cultures, there is no clear-cut relationship between membrane bound nitrate reductase[8] (NAR) synthesis and enzyme activity in complex environments. In their study, synthesis of NAR in sterile soil microcosms occurred after one day in both aerobic and anaerobic environments. The media of a tidal marsh system is much like soil. We postulate, therefore, that populations of facultative denitrifiers in a tidal marsh could have a heterogeneous expression of NAR - some expressing NAR and others not - causing them to behave in aggregate as aerobic denitrifiers, but without the heterotrophic nitrification capacity of constitutive denitrifiers.

Definitive conclusions are premature on the findings of quantitative FISH analysis to the fundamental physiological ecology of a tidal marsh system. What we have done in this study is take a couple of steps out of the "black box" to augment input-output analysis. Continued research is needed to better understand how characterization of the bacterial community in a tidal

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[8] NAR is responsible for the fist step of the denitrification process.

marsh system can provide important insights into process engineering. In our opinion, the insights garnered thus far by genetic probes are valuable when considered in light of other data. Our research continues.

Internal treatment mechanisms of constructed wetlands are poorly characterized, if at all, by "black box" input-output analyses; hence the current deficient status of textbook wetland designs (US EPA, 2000). Wetzel (1993) states that, "The most fundamental characteristic of wetlands is that their functions are almost solely *regulated by microbiota and their metabolism*". Ten years later, the truth of this statement remains. Understanding the biological cast of players gives engineers access to a large body of microbiology literature that contains a wealth of physiological information directly relevant to wastewater treatment. Given the limitations of "black box" wetland treatment design it would be wise for engineers to become familiar with the fundamental physiological ecology of the systems they design. A semi-quantitative design basis relying on strong biology is far more *rigorous* than a design basis comprised of systems of differential equations relying on coefficients derived from statistically confounded (and often poor) data curve fits that have no reference to biological mechanisms. Once the underlying biology of treatment wetlands is decrypted, more powerful and reliable quantitative design tools can be developed.

CONCLUSION

A variety of analyses have demonstrated that a TVF marsh is a treatment system capable of advanced simultaneous nitrification and denitrification. The basic biophysical mechanisms behind nitrification and denitrification have been demonstrated. The design implications of a FISH quantitative characterization of the pilot bacterial community have been tentatively explored. The results of this study, and others in the literature, demonstrate that tidal flow wetland treatment systems substantially outperform conventional wetland treatment systems on smaller treatment footprints. Moreover, the process of *tidal nitration* inherent to tidal flow treatment systems is substantially more energy efficient than activated sludge systems providing advanced biological nitrogen removal.

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