This is not a peer-reviewed article.
Pp. 477-485 in Total Maximum Daily Load (TMDL) Environmental Regulations–II
Proceedings of the 8-12 November 2003 Conference (Albuquerque, New Mexico USA),
Publication Date 8 November 2003.
ASAE Publication Number 701P1503, ed. A. Saleh.

CONSTRUCTED WETLANDS FOR THE REMOVAL OF CONTAMINANTS FROM DAIRY WASHWATER

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ABSTRACT

Surface- and ground-water quality in the Chino-Santa Ana River Basin, California is a major source of drinking water supply for the Los Angeles metropolitan area. This water source is significantly degraded due to intensive dairy operations and the disposal of untreated wastewater into the Chino Basin. Constructed wetlands have been recognized as a treatment option for the removal of high concentrations of contaminants in agricultural waste water prior to land application. The goal of this study was to characterize the fate and transport of chemical contaminants and pathogens in a constructed wetland system and to determine the diversity of ammonia oxidizing bacteria that were responsible for nitrogen mineralization in the wetlands. Water samples were collected weekly for 11 months from two wetlands to determine the efficiency of the treatment system in removal of chemical contaminants, total/fecal coliforms and *Escherichia coli*. Reduction by the treatment was greatest for biological oxygen demand (BOD), suspended solids, chemical oxygen demand (COD), nitrate, and coliforms. There was only moderate removal of total nitrogen and phosphorus. The population of ammonia-oxidizing bacteria showed a higher percentage of *Nitrosospira*-like sequences from the wetland samples, compared to a higher percentage of *Nitrosomonas*-like sequences from manure, feces, raw washwater and facultative pond. These results demonstrate that the wetland system is a natural process dependent upon the development and maintenance of healthy microbial communities for optimal wastewater treatment in reducing dairy waste in the Santa Ana watershed quality

KEYWORDS. Surface- and ground-water, Constructed wetlands, Total/fecal coliforms, *Escherichia coli*, Contaminants, Nitrate, Wash water, Dairy.

INTRODUCTION

The Santa Ana River watershed is located in southern California, encompassing parts of Riverside, San Bernardino and Orange Counties. It is one of the smallest watersheds in the state (2,800 square miles), but has a population of more than 4.5 million people. Although it is one of the most urbanized watersheds in the state it also has the highest density of dairy cows in the nation with as many as 35-40 cows per acre. Currently, 270 dairies with over 386,000 animals operate on 25,000 acres within the Chino Basin portion of the watershed. Although the number of dairies continues to decrease the number of animals is increasing and the resulting impact on water quality is significant. Between 50-75 gallons of water are used to wash down a cow prior to milking generating over 25 million gallons of washwater per day. Current management practices for dairy washwater involve long-term storage in ponds where it is left to evaporate, or percolate into groundwater, or to be sprayed onto crops and/or disposal lands. Dairy washwater is high in dissolved solids, particulate organic matter, ammonia and organically bound phosphorus and nitrogen as well as biological oxygen demand, and other contaminants. Washwater may also contain pathogenic bacteria such as *E. coli* O157:H7, protozoan parasites such as *Cyrptosporidium* and *Giardia,* as well as viruses such as bovine rotavirus. *E. coli* O157:H7 can be transported through storm water after a heavy rainfall washing infected manure into the farming community's well and subsequently contaminating ground water and soil. Water

contamination by *E coli* is becoming common in rural areas of the United States, with up to 40% of tested wells found to be contaminated (US-EPA, 1996). In Walkerton, Ontario, more than 1000 people fell ill and five died of *E. coli* infections following a storm on the 12th of May, 2000 (O'Conner, 2002). Intensification of regional livestock enterprises was named as the likely cause.

Although natural wetlands have existed for ages across the globe, the use of constructed or artificial wetlands built for the improvement of water quality is a relatively new concept of the last two decades. Wastewater from intensive agricultural activities (cattle feedlots and dairies) has significantly higher concentrations of organic matter and nutrients than treated municipal effluent. The high pollutant loads being generated pose particular problems and challenges for the dairy industry since high concentrations of nutrients can contribute to water management problems if wastes are allowed to discharge directly into receiving waters. For this reason, agricultural wastes must be treated prior to disposal. Constructed wetlands in association with stabilization ponds have been suggested as a potential treatment option prior to land application. The extent of water treatment in natural or constructed wetlands depends upon the wetland design, microbial community, and types of plants involved. The bulk of the water quality improvement in natural and constructed wetlands is attributed to bacteria (Gersberg et al., 1986).

It has long been recognized that certain microbial groups in animal waste are responsible for breaking down various organic compounds and suppressing pathogens in solid waste or water. The diversity of microorganisms in the wetland environment may be very critical for the proper functioning and maintenance of the system. One such group of bacteria involved in different biological/chemical transformations of organic compounds in wastewater is the chemolithotrophic ammonia-oxidizing bacteria. These bacteria are responsible for the first, rate-limiting step in nitrification where ammonium (NH_4) is transformed to nitrite (NO_2) and to nitrate (NO_3) during natural nitrogen cycling (Jetten et al., 1997; Paul and Clark, 1989). Oved et al. (2001) studied the effects of urban sewage effluent on the community composition and function of ammonia-oxidizing bacteria in soil using DGGE of PCR-amplified fragments of the *amo*A gene. They found a significant and consistent shift in the population composition of ammonia-oxidizing bacteria in soil irrigated with effluent dominated by *Nitrosomonas*-like populations, while the non-effluent soil was dominated by *Nitrosospira*-like strains. Our first objective was to determine the effectiveness of the wetland treatment technology in reducing contaminants in dairy waste effluent. The second objective was to examine the composition of ammonia-oxidizing bacteria in manure, feces, and dairy washwater prior to and after treatment in subsurface wetlands, and to determine how the composition of the community influences the final wastewater effluent quality.

MATERIALS AND METHODS

Wetland design and sampling regime

The wetland design consisted of two sub-surface horizontal flow beds (60 x 10 x 1 m) operating in parallel, and a raw and facultative pond for central collection of the washwater prior to treatment (Ibekwe et al., 2002). Coarse and finer gravel were place up to a depth of 1 m and were graded across the length of the flow bed. The first 6 m length of the wetlands contained coarse and fine gravel to act as a sink for particulate matter. The next 6 m contained a bed of reeds (*Phragmites communus*) with a shallow, dense root system. The root system acted as a physical filter to remove suspended matter. The remaining 48 m of the wetland contained bulrush (*Scirpus validus*).

Wetland 1 was an end-loading design, where washwater entered through multiple inlets onto the coarse gravel, reeds and bulrush, then drained into the collection box at the end of the basin. Wetland 2 was a side-loading basin, where washwater entered through multiple inlets along both sides of the wetland, passed through a narrow gravel bed containing reeds and bulrush, and was collected through a perforated pipe along the center of the wetlands that drained into the collection box. The combination of open gravel and specific plants was used for the initial

removal of suspended solids and subsequent nitrification of the wastewater in the micro-aerobic zone surrounding the roots of the bulrushes. The overall design of the wetlands was based on studies carried out by Gersberg et al. (1986), with some modifications for higher BOD, nutrient levels, and suspended solids.

Monthly samples were collected from six locations: the wastewater lagoon, facultative pond, and wetlands 1 and 2 influents and effluents. Fresh cow and calf fecal samples and manure samples were collected for microbial analysis. Weekly waste water samples were collected for chemical analysis and determination of the quality of the final effluent water. All samples were collected between December 2000 and September 2001. Weekly samples were analyzed for total Kjeldahl nitrogen (TKN), ammonium nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N), biochemical oxygen demand (BOD), total suspended solids (TSS), orthophosphate (PO_4-P), pH, potassium (K^+), sodium (Na^+), chloride (Cl^-), total dissolved solids (TDS), chemical oxygen demand (COD)-filtered and unfiltered, and fecal and total coliform bacteria. All analyses were done using protocols from the Standard Methods of the American Public Health Association (1995). Heterotrophic bacteria, *E. coli* and *E. coli* O157 were enumerated by culture methods on tryptic soy agar (TSA), Sorbitol MaConkey agar (SMAC) and with cefiximine and tellurite (CT-SMAC) agar, respectively.

DNA extraction from soil and effluent and DGGE analysis of ammonia oxidizers

Total bacterial DNA was extracted from 500 mg of fecal or manure samples and from pellets of concentrated influent or effluent samples (centrifuged at 3,000 × g for 10 min). DNA was extracted using theUltraClean Fecal and Water DNA kits (MO BIO, Inc., Solana Beach, CA), according to the manufacturer's protocol. The diversity of the ammonia-oxidizing bacteria in the samples was determined by PCR with *amo*A primers targeting a partial stretch of the genes that encode for the active-site polypeptide of ammonia monooxygenase (Rotthauwe et al., 1997). PCR products were analyzed by DGGE with a linear chemical gradient ranging from 30 to 60% denaturant. DNA was visualized after ethidium bromide staining and major bands were excised for identification of ammonia-oxidizing bacteria. Bands were placed into sterilized vials with 20 µl of sterilized distilled water and treated as described above. A total of 10 µl of eluted DNA was used as template with *amo*A primers without the GC clamp for re-amplification. PCR products were purified with the QIAquick PCR purification Kit and cloned as above.

Statistical analysis and analysis of DGGE bands

Data analyses of nutrient concentrations were performed using SAS (SAS, 1988). Analysis of variances, means and standard deviations for the individual nutrient in triplicate samples were determined to compare the concentration of each nutrient in each sample over the sampling period.

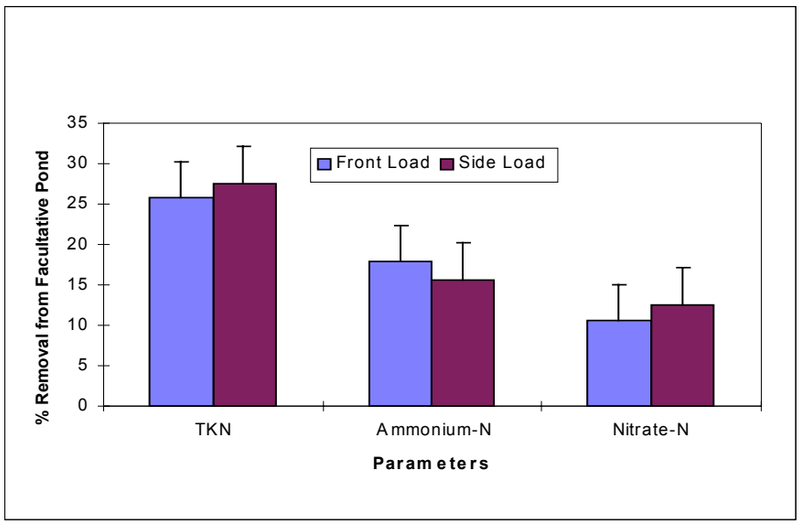
Phylogenetic analysis

Sequence analyses were done using the BLAST database (National Center for Biotechnology Information:www.ncbi.nlm.nih.gov). Partial *amo*A gene sequences were aligned with parts from the complete *amo*A gene sequences of ammonia oxidizing bacteria obtained from the BLAST gene bank.

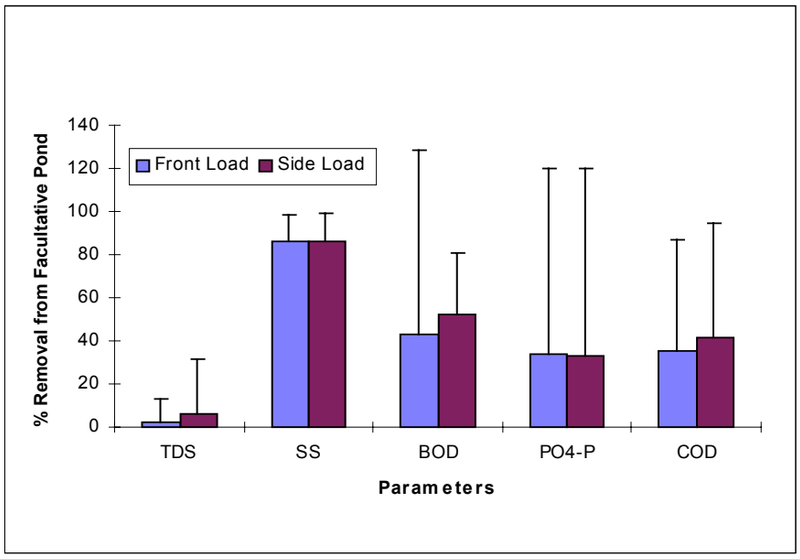
RESULTS AND DISCUSION

Wastewater constituents

Wastewater samples from wetlands were analyzed for TKN, PO_4-P, K, NH_4-N, NO_3-N, BOD, TDS, pH, TSS, Na^+, Cl^-, COD, total coliform bacteria, total *E. coli* and *E. coli* O157. The TKN levels in raw washwater were 150- 250 mg l^{-1} with ammonium levels of 100 – 200 mg l^{-1}, and were reduced 25% and 16% through wetlands treatment, respectively (Fig. 1 A). The total nitrogen in the raw washwater was removed by an average of 25%. Although the BOD of the raw washwater ranged from 50-350 mg l^{-1} and steadily increased



A



B

FIG.1 A&B. Percent removal of major chemical components in wetland. Analyses were done on weekly samples. Results are presented as an average removal rate for the eleven months that samples were collected.

as the ambient temperature increased in the spring and summer, reductions through wetlands treatment remained constant at an average of 73% (Fig 1B). The suspended solids ranged widely (100-1100 mg l^{-1}) in the raw water, while levels in wetlands effluent were consistently lower with an average drop of 91%. COD data were collected to determine the amount of oxidizable organic and inorganic matter in the raw and treated washwater. There was a 38% drop in COD-filtered, and a 33% decrease in orthophosphate (Fig. 1 B). The pH remained close to neutral (range of 7-8) throughout the system. The removal efficiencies for TSS, BOD and total/fecal coliform bacteria were similar to those described by Gersberg et al. (1986) in wetlands treatment of domestic wastewater. Wetlands treatment achieved a 99% decrease in total coliform bacteria and a 99.9% decrease in fecal coliform bacteria between the raw washwater pond and the average wetland effluent (Fig. 2). This is significant both from the standpoint of surface runoff, as well as potential

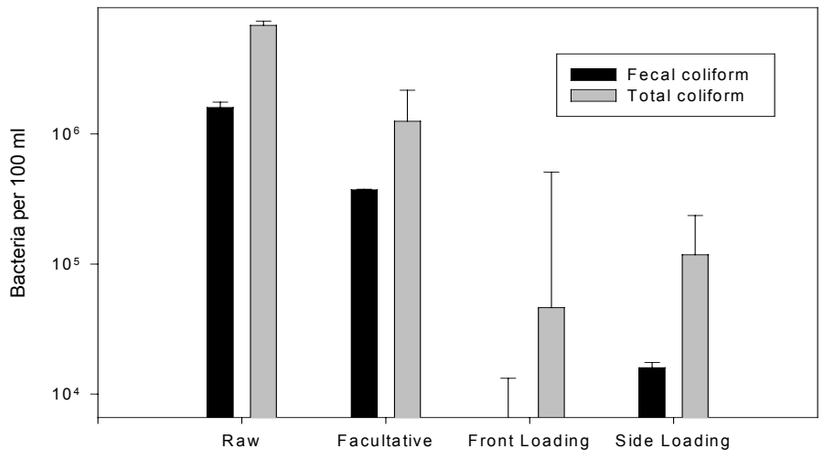


FIG.2. Changes in fecal and total coliform concentrations in and out of wetlands. Values shown here are based on the geometric mean from four samples averaged from each of the sampling months. Concentration of bacteria was determined from 100 ml of samples taken at different locations in the wetlands.

airborne pathogens released during the spray irrigation of raw washwater on disposal lands. There were no significant differences in the treatment efficiencies between the wetlands.

Dairy washwater is approximately three to five times higher in major constituents (e.g. nitrogen, phosphorus, BOD, etc.) than domestic raw sewage. Even with these constraints, the results were quite promising. The greatest success in treatment was for BOD, suspended solids, filtered and unfiltered COD and coliforms. There was moderate success in the removal of nitrogen and phosphorus, as is the case with most subsurface wetlands due to higher levels in the influent to the wetland system. There was little change between the influent and effluent concentrations of the conservative ions, such as chloride, potassium and sodium. These ions are conservative constituents that vary little, if at all, due to biological activity. Because of their conservative nature, they are useful tools for determining retention and travel times of washwater through the wetlands system.

Concentration of bacteria in different matrices

Bacterial concentrations in the different matrices associated with the wetlands were determined between December, 2000 and September, 2001. The number of heterotrophic bacteria from different matrices ranged from 1.9 x 10^5 CFU ml^{-1} in the wetland 1 effluents to 6.9 x 10^8 CFU g^{-1} in the manure samples. Total *E. coli* concentrations in the different matrices, as determined by culture method on SMAC agar ranged from 7.5 x 10^2 CFU ml^{-1} (wetland effluent) to 6.5 x 10^7 CFU g^{-1} (manure). There was a three to four-log reduction in *E. coli* concentrations between the raw washwater and the final effluent water (Fig. 3). The concentrations of *E. coli* O157 on CT-SMAC from the different matrices ranged from 48 CFU ml^{-1} in wetland 1 effluents to 1.1 x 10^7 CFU g^{-1} in the manure samples (Fig. 4). All samples were collected in triplicate and combined after enumeration for average values. Overall reduction of three to four-log of total *E. coli* and *E. coli* O157 in this wetland demonstration project was observed. The most efficient unit of the project in removing *E. coli* and *E. coli* O157 was the facultative pond. The effects of seasonal variations on the concentration of *E. coli* and *E. coli* O157 samples were grouped and mean separation was carried out by the least-significant difference (LSD) test. The concentrations of *E. coli* and *E. coli* O157 were not significantly different among the six sampling points in February, April, and September, but were highly significant in July and

August. On the average, the highest concentration of *E. coli* and *E. coli* O157 was recorded in July with the lowest in September at all the sampling points in the wetlands.

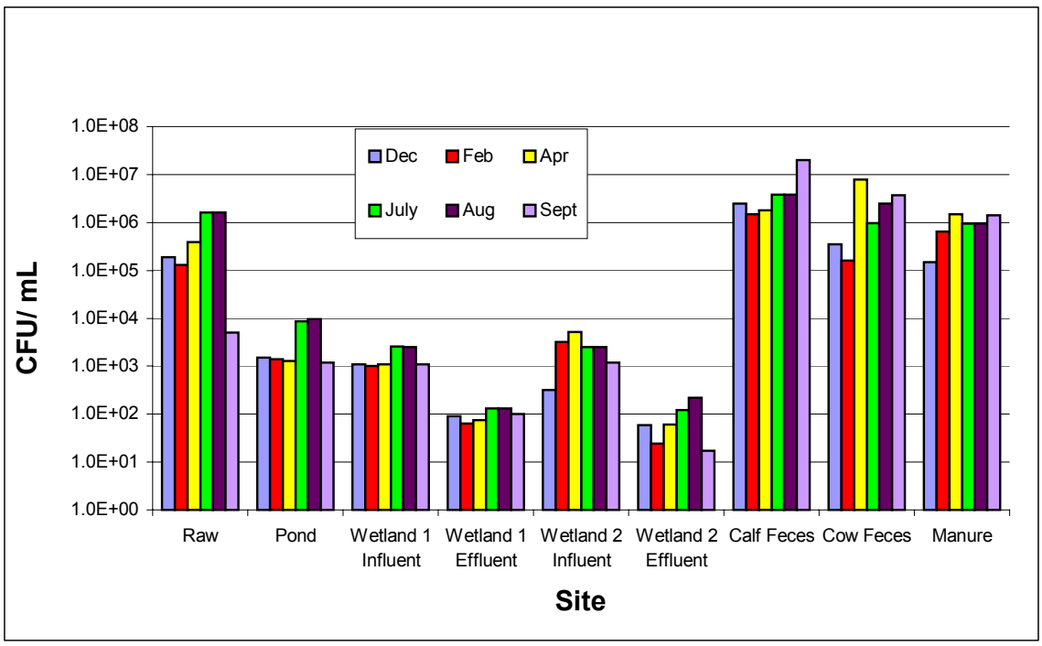


FIG. 3 Concentration of *E. coli* detected in environmental samples by culture methods. All solid samples were from calf feces, cow feces, manure, and the liquid waste were from facultative pond (pond), raw pond (Raw), wetland 1 effluent, wetland 1 influent, wetland 2 effluent, and wetland 2 influent. Total *E. coli* (CFU 100 ml^{-1}) from different samples determined on SMAC media.

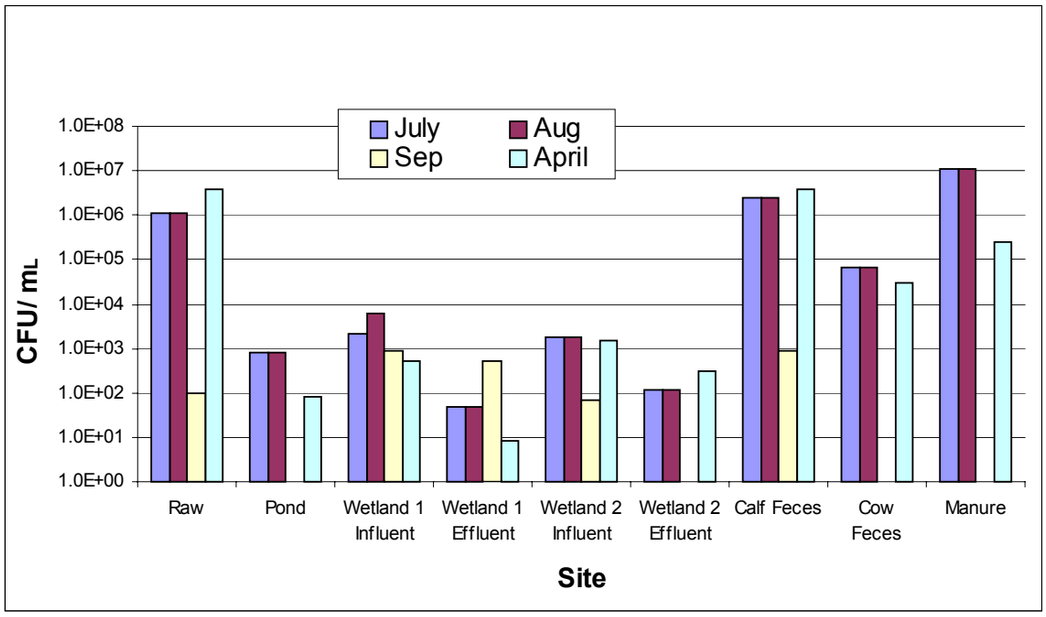


FIG.4. Concentration of *E. coli* O157 detected in environmental samples by culture methods. All solid samples were from calf feces, cow feces, manure, and the liquid waste were from facultative pond (pond), raw pond (Raw), wetland 1 effluent, wetland 1 influent, wetland 2 effluent, and wetland 2 influent. *E. coli* O157 (CFU 100 ml^{-1}) was determined on CT-SMAC media.

Determination of ammonia-oxidizing bacteria composition by *amo*A genes

The functional gene encoding the α subunit of ammonia monooxygenase (*amo*A) was characterized in wetland bacterial community. A total of nine samples and two positive controls

were used for the DGGE analysis (Fig. 5). Four distinct dominant banding patterns from the nine samples and the controls were determined. *Nitrosomonas* ATCC 19178 migrated to the top portion of the gel and *Nitrosospira* ATCC 25196 migrated to the lower portion of the gel. Sequence analysis revealed that the lower bands were phylogenetically related to *Nitrosospira* species, while the upper bands were phylogenetically related to *Nitrosomonas* species (Fig. 5). Two out of three *Nitrosomonas*-like sequences obtained from the wetlands samples were closely related *Nitrosomonas europaea* and the remaining one to *Nitrosomonas mobilis* cluster. All sequences related to *Nitrosomonas* obtained from the wetlands and manure samples belonged to the *N. europaea-Nitrosococcus mobilis* cluster.

The *amoA* PCR products (primers amoA-1F and amoA-2R) retrieved from the samples were used for the generation of *amoA* cloned libraries of the wetland ammonia-oxidizing bacteria. Phylogenetic analysis of 30 cloned samples demonstrated that all clones contained *amoA* sequences affiliated to the beta-subclass ammonia-oxidizing bacteria (Table 1). *Nitrosospira*-related sequences were detected in all the samples analyzed in this study. *Nitrosomonas*-related sequences were detected in manure, feces, and the raw wastewater holding pond and had fewer sequences in the wetland samples. The analyses of *Nitrosospira*-related sequences showed that the majority of the wetland samples were phylogenetically related to *Nitrosospira*. These samples were closely related to three main groups, with most of them closely related to uncultured bacterium NAB-8-C11, the second group was closely related to *Nitrosospira* sp. Nsp12 and the third to *Nitrosospira tenuis*.

Table 1. Bacteria identified from cloning *amo*A genes.

BLAST Gene Data Base match	Sequence similarity (%)	Gel position	Accession no.
Nitrosomonas sp. WH-2 16S	100	007	AF338211
Nitrosospira multiformis	100	005	AY123807
Uncultured beta proteobacterium clone 4.5E	100	006	AF266816
Nitrosospira sp. Nsp2	92	005	AJ298719
Nitrosomonas sp. WH-2	93	004	AF338211
Nitrosomonas europaea	100	002	AF058692
Nitrosospira 25196	100	001	U76553
Nitrosomonas 19178	100	003	AFO16003

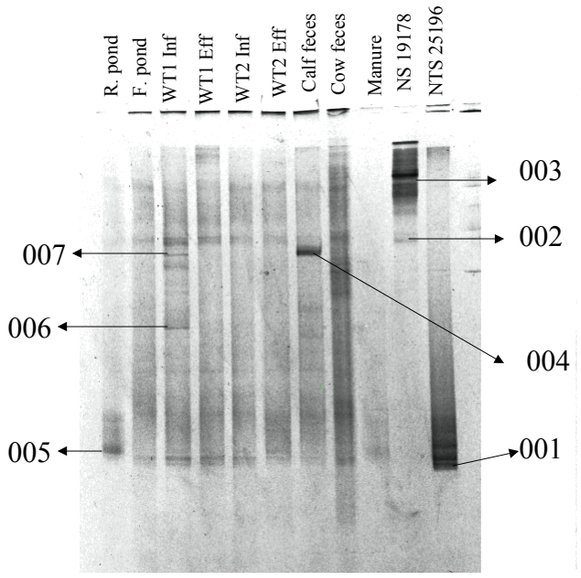


Fig 5

FIG. 5. DGGE analysis of *amo*A fragments obtained from feces, manure and wetland samples. Similar samples were pooled from all sampling dates (December 2000 to September 2001) for analysis. Nine samples were used for the final analysis plus the control samples for *Nitrosomonas* ATCC (NS 19178) and *Nitrosospira*

ATCC (NTS 25196). F and R represent facultative pond and raw pond, respectively, WT1 and 2, Inf or Eff represent wetland 1 and 2 influent or effluent.

CONCLUSIONS

One of the major objectives for the establishment of constructed wetlands is to use the final effluent for irrigation and/or for disposal into other bodies of water. In this study, the wetland effluent was more suitable for on-site reuse and would reduce the amount of contaminants entering groundwater supplies as a result of percolation of the washwater stored in ponds and sprayed on disposal lands. The removal of the main pollutants from the dairy washwater has a beneficial impact on the surface and groundwater in the Chino Basin, and, in turn, would benefit the quality of water leading into the Santa Ana River and the Orange County groundwater basin. This wetland project serves as an innovative model for waste management for the dairy industry and other confined animal facilities. Providing a cost-effective, low-maintenance process that can be independently built and managed, wetland treatment systems throughout the Chino basin would have a significant long-term impact on the quality of the ground and surface water supply.

ACKNOWLEDGEMENTS

We thank Ms Katherine O'Connor, project director of the Chino dairy wetlands and the members of staff of the Orange County Water District who were instrumental in the construction of the wetlands, especially the Technical Advisory Committee. This research was supported by the 206 Manure and Byproduct Utilization Project of the USDA-ARS. Mention of trademark or propriety products in this manuscript does not constitute a guarantee or warranty of the property by the USDA and does not imply its approval to the exclusion of other products that may also be suitable

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