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Effectiveness of treatment and diversity of microbial populations within a constructed wetland treating wastewater

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**EFFECTIVENESS OF TREATMENT AND DIVERSITY OF MICROBIAL
POPULATIONS WITHIN A CONSTRUCTED WETLAND TREATING WASTEWATER**

by

Jolene M Friedland

**Thesis submitted to the Davis College of Agriculture, Forestry, and Consumer Sciences
at West Virginia University
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for the degree of**

**Master of Science
in
Plant and Soil Sciences with a concentration in Environmental Microbiology**

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ABSTRACT

EFFECTIVENESS OF TREATMENT AND DIVERSITY OF MICROBIAL POPULATIONS WITHIN A CONSTRUCTED WETLAND TREATING WASTEWATER

by Jolene M Friedland

Constructed wetlands are used as alternative on-site wastewater treatment systems. Microbiological dynamics in constructed wetlands are essential to adequate functioning and efficiency of these systems. The efficacy of wastewater treatment by a large subsurface flow wetland receiving wastewater from a multi-user facility was examined. Real-time monitoring with telemetry was combined with measurement of standard wastewater parameters such as biochemical oxygen demand (BOD₅), total suspended solids (TSS), and enumeration of fecal coliforms. Passage of wastewater through the wetland significantly reduced standard wastewater parameters in effluent samples. Heterotrophic bacterial diversity was examined using community level physiological profiling (CLPP) and molecular characterization of 16S ribosomal RNA genes by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). Principal component analysis (PCA) indicated distinct grouping between the influent and effluent wastewater populations while DGGE analysis indicated distinct groupings according to cell size.

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GENERAL INTRODUCTION

Constructed wetlands are now in use as alternative on-site wastewater treatment systems. Constructed wetlands can reduce wastewater pollutants from a variety of sources including individual households, small towns, urban storm water runoff, agricultural point and nonpoint sources, landfill leachate, mine drainage, and water containing organic micropollutants (2). Effectiveness of wastewater treatment within constructed subsurface flow wetland systems is usually evaluated by estimating removal rates of organic matter, suspended solids, nutrients, and bacteria (especially *Escherichia coli* and other coliforms) (3). Bacteria play a central role in the wastewater treatment process within constructed subsurface flow wetlands. They dominate both in numbers and biomass, and regulate mineralization and immobilization of organic and inorganic nutrients (1). The physiology, survival strategies, and community structure of bacterial populations in constructed subsurface flow wetland systems have not been extensively studied. A better understanding of bacterial dynamics in these systems will contribute to improved system design, functioning, and efficiency of constructed subsurface flow wetland systems.

In the current research, a polyphasic approach was used to evaluate treatment effectiveness and maintenance compliance of an on-site subsurface flow constructed wetland serving a large multi-user facility. Standard analyses of physical and chemical parameters, total heterotrophic bacteria, and indicator organisms were performed. The objective was to determine whether passage of primary clarified wastewater through the constructed wetland system resulted in regulatory compliance and if significant differences in treatment effectiveness were observed between winter and summer months. *In-situ* real-time telemetry (continuous automatic measurement and transmission) also was used to measure hydraulic loading rate of primary clarified wastewater to the wetland system, effluent discharge from the wetland system to the soil drain field, pH, dissolved oxygen (D.O.), wetland water temperature, and ambient air temperature. The objective was to gain information about changes in hydrology and *in-situ* physical and chemical parameters of the on-site wastewater treatment system and to explore possible correlations of telemetry with results from conventional sampling. Community-level physiological profiles (CLPP) of the total cell (TC), large cell (LC), and small cell (SC) fractions

of heterotrophic bacteria community were determined using BIOLOG® ECO plates. In addition, the genetic diversity of the bacterial community was determined by denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified 16S rRNA genes. The objective was to compare functional diversity and genetic diversity of the bacterial community within the wetland system.

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CHAPTER 1: REAL-TIME MONITORING AND SEASONAL EFFECTIVENESS OF A SUBSURFACE FLOW CONSTRUCTED WETLAND TREATING WASTEWATER FROM A LARGE MULTI-USER FACILITY

INTRODUCTION

According to the 1990 U.S. Census, more than 1.1 million households nationwide lack public sewerage, a septic tank, or a cesspool (26). In Appalachia and locally in West Virginia, many households and rural communities lack centralized wastewater collection and treatment facilities due to the mountainous terrain, low population densities or a lack of financial resources (9). In this case, where centralized systems are unavailable, a septic tank with a soil drain field is the most common alternative. However, installation of a septic tank with a soil drain field capable of effective treatment is not always feasible due to poor site conditions such as steep topography, low soil percolation, and limited availability of land. In the U.S., where decentralized systems serve approximately 25% of the population, septic tank effluent is the most frequently reported source of pathogenic groundwater contamination (27). Therefore, there is a need for alternative and cost-effective on-site treatment systems.

Subsurface flow constructed wetlands currently are used as alternative on-site treatment systems in West Virginia. Constructed wetlands are permitted and regulated by the West Virginia Department of Environmental Protection (WVDEP), Division of Water and Waste Management (DWWM). In West Virginia, constructed wetlands currently are classified as experimental treatment systems due to the lack of approved design standards and the limited information on operations and problems with existing installations. The WVDEP average monthly discharge limitations for alternative systems are 30 mgL^{-1} BOD₅, 30 mgL^{-1} TSS, and 200 CFU 100 mL⁻¹ fecal coliforms; maximum daily discharge limitations are 60 mgL^{-1} BOD₅, 60 mgL^{-1} TSS, and 400 CFU 100 mL⁻¹ fecal coliforms; and instantaneous maximum discharge limitations are 75 mgL^{-1} BOD₅, 75 mgL^{-1} TSS, and 500 CFU 100 mL⁻¹ fecal coliforms. Additionally, the pH of the discharge must be in the range of 6.0 to 9.0 standard units. These

standards often are not met by alternative treatment systems, including both constructed wetlands and aerobic treatment units (ATUs) (16,20,25).

Constructed wetlands have been used to treat wastewater sources ranging in size from individual households to small towns (5,6). Subsurface flow wetlands consist of channels or basins that contain substrate (predominantly sand, gravel, or mixtures) that support growth of emergent vegetation such as cattails (*Typha sp.*), bulrushes (*Scripus sp.*), reeds (*Phragmites sp.*), rushes (*Juncus sp.*) and sedges (*Carex sp.*) (6,18,23). Primary clarified wastewater effluent, often from a septic tank, discharges a few inches below the surface of the substrate. Wastewater flows horizontally through the substrate, is collected in an outlet pipe, then released to a soil drain field, or disinfected with chlorine prior to direct discharge into a receiving stream. Subsurface flow wetlands decrease mosquito breeding, objectionable odors, and the possibility of human/animal contact with partially treated wastewater.

Wastewater treatment efficiency within constructed subsurface flow wetland systems usually is assessed by estimating removal rates of organic matter, suspended solids, and coliform bacteria (12). Pollutants are removed by a variety of mechanisms, including both aerobic and anaerobic microbial processes, sorption, sedimentation, volatilization, and chemical transformations. Organic compounds are removed through settling, entrapment of particle matter in void spaces, and microbial growth on the substrate surface and plant roots and rhizomes (11). Bacteria are removed by physical factors—filtration, sedimentation, and aggregation; biological mechanisms—antibiosis, ingestion by nematodes, protozoan, lytic bacteria and bacteriophage, and chemical factors—oxidation, adsorption, and exposure to toxins given off by other microorganisms and plants (21).

Bacteria play a central role in the wastewater treatment process within constructed subsurface flow wetlands. They dominate both in numbers and biomass, and regulate mineralization and immobilization of organic and inorganic nutrients (1). Enumeration of fecal indicator bacteria (coliforms) always has been a critical part of sanitary evaluation (29). Two-thirds of all states use fecal coliforms to monitor the quality of both fresh and marine waters (29). *E. coli* also has been demonstrated to be a specific indicator for the presence of fecal contamination (13).

Enterococci also are useful indicators of microbiological water quality since they are common inhabitants of the intestinal tracts of humans and lower animals (15). Total heterotrophic bacteria recently have been implicated as potential opportunistic pathogens in drinking water (24).

In this research, studies were performed to evaluate treatment effectiveness and maintenance compliance of an intensely utilized subsurface flow constructed wetland treating wastewater from a large multi-user facility. Analyses of physical and chemical parameters, total heterotrophic bacteria, and indicator organisms were performed. The objectives were to determine whether passage of primary clarified wastewater through the constructed wetland system consistently resulted in significant treatment and whether significant differences were observed between winter as compared to summer months. During summer months *in-situ* real-time telemetry (continuous automatic measurement and transmission) also was used to measure hydraulic loading rate of wastewater to the wetland system, effluent discharge from the wetland system to the soil drain field, pH, dissolved oxygen (DO), wetland water temperature, and ambient air temperature. Possible correlations were examined between telemetry measurements and those obtained with conventional sampling.

MATERIALS AND METHODS

Wetland System

The system investigated was a constructed subsurface flow gravel wetland (~11.89 m x 5.49 m x 0.45 m) treating primary clarified wastewater from a two-compartment septic tank (Tank 1 5678 L and Tank 2 1893 L) discharging from a multi-user church facility (Figure 1). The second compartment of the septic tank acted as a dosing tank for the wetland. The number of users attending the facility varied throughout the week and also throughout the study period; however, the maximum number of users was always on Sunday. It was estimated approximately 1300 people were present on Sundays, with numbers during the remainder of the week ranging from 10-250 depending on on-going programs and functions. The wetland was constructed with 1.3 cm gravel (approximately 44% porosity - based on field testing), lined with a 50 mm flexible liner, and the area surrounding the wetland was constructed with larger stone to limit overland runoff to the wetland. The slope of the wetland was approximately 1% from the inlet to discharge end. The wetland water level was maintained at a constant level of approximately 18 cm. Approximately 40% of the wetland was planted with reed (*Phragmites sp.*) and iris (*Iris sp.*). For aesthetic purposes, one side of the wetland was nearly completely covered with reed and iris plants while the other side contained few plants. Treated effluent flowed by gravity from the wetland to a valve box that led to a holding tank (1893 L). The holding tank dosed a shallow low-pressure pipe (LPP) drain field (5 narrow 0.25 m gravelless pipe trenches) for final disposal. The advantages of this type of drain field include uniform small doses to the entire adsorption area that in turn promotes unsaturated flow and also results in consistent drying/reaeration periods between doses (14).

Telemetry Configuration

The wetland system was equipped with telemetry instruments to evaluate the potential to continuously monitor in real-time *in-situ* parameters as a means to monitor treatment effectiveness and maintenance compliance. Parameters monitored with *in-situ* real-time telemetry included pumping duration of the septic tank to the wetland and of the holding tank

to the drain field (measured using floats located in the pump chambers and a general purpose relay (Allen-Bradley, Milwaukee, WI)), pH of the first compartment of the septic tank (Q25P pH Sensor, Quantum Analytical Instruments, Collegeville, PA), dissolved oxygen in the valve box at the discharge end of the wetland (Q25D Dissolved Oxygen Sensor, Quantum Analytical Instruments, Collegeville, PA), wetland water temperature (Oxyguard International, Denmark) and ambient air temperature (Oxyguard International, Denmark) (Figure 1). Telemetry readings were taken in three-minute intervals and transferred using a remote monitoring system (Sensaphone® 2000, Sensaphone, Ashton, PA) via a phone line to a computer and stored as text (.txt) files. Telemetry readings recorded June 1, 2004 through August 30, 2004 are included in this paper. Pumping duration of the septic system was used to calculate hydraulic loading rate (HLR) and hydraulic retention time (HRT), while pumping duration of the holding tank was used to calculate soil drain field loading rate. When the pump was activated an electrical signal was sent by the floats via the relay to the Sensaphone® system located in the telemetry panel. HLR was calculated using the following equation: $HLR = \bar{O}(PD \cdot 2.72)$, where PD = pumping duration (sec) and 2.72 = pumping rate ($L \text{ sec}^{-1}$). HRT was calculated as follows: $HRT = ((11.89 \text{ m} \cdot 5.49 \text{ m} \cdot 0.23 \text{ m} \cdot .44 \cdot 1000 \text{ L m}^{-3}) / HLR \text{ (L day}^{-1}))$, where 11.89 m = length of wetland, 5.49 m = width of wetland, 0.23 m = depth of water in wetland, and .44 = porosity of wetland. Soil drain field loading rate was calculated as $LPLR = \bar{O}(PD \cdot 0.95)$, where PD = pumping duration (sec) and 0.95 = pumping rate ($L \text{ sec}^{-1}$).

Sampling Methods

A total of 12 grab water samples were collected between 8 and 12 a.m. during the winter period (January 2004 - March 2004) and a total of 34 water samples were collected at similar times during the summer period (June 2004 – August 2004). Influent samples were taken from the second compartment of the septic tank (primary clarified wastewater) and effluent samples were taken from a 15 cm diameter PVC temporary monitoring well installed near the discharge point of the wetland. Samples were collected in sterile 1-liter Nalgene® plastic bottles and packed on ice in an insulated cooler during transportation to the laboratory, where they were analyzed within 24 hours.

Physical and Chemical Analyses

Immediately following sample collection, field measurements of dissolved oxygen, electrical conductivity, pH, and temperature were performed using a YSI Model 556 MPS Meter (Yellow Springs Instruments, Yellow Springs, OH) following standard procedures of the American Public Health Association (2). Upon arrival to the laboratory, samples were refrigerated until processing. Five-day biochemical oxygen demand (BOD₅) and total suspended solids (TSS) were measured following standard methods (2). Dissolved oxygen values from day five of the BOD₅ incubation were corrected to adjust for any decreases in oxygen of the dilution water blanks over the five-day incubation period.

Bacteriological Analyses

Samples were analyzed for fecal coliforms, *Escherichia coli*, enterococci, and total heterotrophic bacteria by a modified standard method (2) using a Spiral Biotech Autoplate 4000 Automated Spiral Platter (Spiral Biotech, Inc., Norwood, MA) and the membrane filtration technique (2) using 0.45 µm Millipore HA filters (Millipore Corp., Bedford, MA). Serial dilutions were performed or appropriate volumes were filtered to obtain plates with 20-300 colonies (modified standard method) or 20-200 colonies (membrane filtration technique). Plates were incubated for 24 hours at 44.5°C on M-FC medium (Difco, Detroit, MI) for fecal coliforms, 24 hours at 44.5°C on EC-MUG (Difco, Detroit, MI) for *E. coli*, 48 hours at 35°C on m-enterococcus medium (Difco, Detroit, MI) for enterococci, and 48 hours at 35°C on R2A medium (Difco, Detroit, MI) for total heterotrophic bacteria.

Statistical Analyses

All statistical analyses were performed using the SAS Statistical Software Package (SAS Institute Inc., Cary, NC). Microbiological data were log₁₀ transformed to obtain geometric means prior to statistical analyses. Differences between physical, chemical, and bacteriological measurements in winter as compared to summer were evaluated using Proc GLM with LSMeans and the tdiff option. Pearson Product Moment Correlation (Pearson's correlation) was used to evaluate correlations between *in-situ* real-time telemetry measurements and

physical, chemical, and bacteriological analyses. Unless otherwise noted, significance was based on a P value of less than or equal to 0.05.

RESULTS

Comparison of Telemetry with Conventional Sampling

Telemetric reports of pumping duration during summer months were used to calculate hydraulic loading rate from the septic tank to the wetland, and to estimate hydraulic retention time within the wetland. The mean hydraulic loading rate to the wetland ranged from 2567 to 2702 L day⁻¹ depending on the averaging period employed (Table 1). Daily estimates of hydraulic loading rates were quite variable ranging from 356 L day⁻¹ to 12913 L day⁻¹, and cycled in a regular pattern on a weekly basis (Figure 3, 5). Highest hydraulic loading rates and lowest hydraulic residence times always were observed on Sundays, consistent with expected peak occupation of the multi-user facility. Average hydraulic retention time ranged from 3.5 to 6.6 days (Table 1) with daily estimates of hydraulic residence time varying from 0.5 to 18.5 days (Figure 3, 5). Estimates of hydraulic loading do not take precipitation into account and assume that all water entering the wetland from the septic tank is dosed to the LPP drain field. Therefore, actual retention time could be either overestimated or underestimated. Discharge rates from the wetland to the drain field also were calculated from telemetric reporting of pumping duration. Average discharge rates ranged from 841 to 1575 L day⁻¹ (Table 1). Direct comparison of discharge rates with hydraulic loading is complicated by storage of the wastewater in a holding tank prior to dosing of the LPP drain field. Average discharge rates generally were lower than hydraulic loading rates with differences probably reflecting evaporation or evapotranspiration. Water temperatures within the wetland were 21.2 to 23.6 C, compared with air temperatures ranging from 22.1 to 25.3 C (Table 1, Figure 2, 4).

Telemetry values were compared with daily measurements obtained using conventional water analyses over a one-week period, and with samples obtained over the entire summer.

Telemetric values for pH and DO exhibited some variation over the summer sampling period, with daily averages ranging from 5.8 to 9.3 and 0.25 mg L⁻¹ to 1.48 mg L⁻¹, respectively (Table 1, Figure 2). Telemetric measurements of pH and DO were consistently lower than values obtained by conventional analyses performed daily for one week (Table 2) and for the summer period (Table 3, Table 2). Conventional pH measurements exhibited an average for one week

of 7.45 and a summer average of 7.15 (Table 3). Differences between telemetric and conventional measurements of pH most probably reflect sampling location in the septic tank (first compared with second settling compartment, respectively). However, differences in measured DO cannot be explained in this manner since conventional field analyses, which exhibited a one week average of 2.45 mg L^{-1} (Table 2) and a summer average of 1.63 mg L^{-1} (Table 3), were performed in a sampling well immediately adjacent to the one housing the permanently installed telemetric oxygen probe. Periodic biofouling of the membrane and localized oxygen depletion caused by the probe electrode during intermittent low water flow are likely explanations for the lower average DO values obtained with the telemetric probe.

During the week-long sampling period a significant reduction between influent and effluent BOD_5 (92.9%) and TSS (77.2%) was observed (Table 2). The range of daily BOD_5 in the influent was 157.3 mg L^{-1} to 207.8 mg L^{-1} and in the effluent was 2.9 mg L^{-1} to 26.8 mg L^{-1} (Figure 7). The range of daily TSS in the influent was 20.0 mg L^{-1} to 43.3 mg L^{-1} and in the effluent was 4.4 mg L^{-1} to 14.4 mg L^{-1} (Figure 7). Effluent BOD_5 was highest on Sunday and Monday, which coincided with the highest spike in hydraulic loading rate and lowest hydraulic retention time (Figure 6). Effluent TSS peaked on Sunday, and again on Wednesday reflecting a second smaller peak in hydraulic loading (Figure 6, 7). Values for other measured parameters such as pH, DO, and microbial counts were relatively consistent regardless of sampling day, and appeared to be only moderately affected by the observed variations in hydraulic load. Conductivity was significantly reduced by 48% (Table 2), but exhibited no obvious temporal trend with respect to hydraulic loading. Microbial populations also were consistently and significantly reduced (range 2.0 to 3.2 $\log_{10}$ reduction) during passage through the wetland irrespective of sampling day (Table 2).

Seasonal Performance of the Wetland Treatment System

Conventional analyses of physical and chemical parameters measured in water samples generally indicated that wetland treatment of wastewater was improved in summer compared with winter months (Table 3). The temperature of the influent wastewater differed, averaging 8.9 and 19.4 $^{\circ}\text{C}$ in winter and summer, respectively. During passage through the wetland, wastewater cooled to an average 5.7 $^{\circ}\text{C}$ during winter months and heated to an average 22.1

°C in summer. Other seasonal differences in influent wastewater included higher average pH and DO; and lower average conductivity, BOD₅ and TSS in winter months compared with summer (Table 3). Wetland treatment resulted in significantly decreased pH and increased DO during winter, but not summer. Reduction in average BOD₅ was observed both in winter (64.7%) and summer (89.5%), however only the summer reduction was statistically significant due to the variability of winter BOD₅ measurement. Significant reductions were observed for TSS (62.9 and 82.9 %) during winter and summer, respectively.

Fecal coliforms, *E. coli*, enterococci, and total heterotrophic bacteria also were enumerated during the seasonal sampling regime (Table 4). No differences between numbers in winter and summer influent were observed, with the exception of enterococci, which were significantly greater in winter samples. Significant differences between influent and effluent enumerations, with log₁₀ reductions ranging from 1.6 to 3.5, were observed for all bacterial types regardless of season. Greater reductions were observed in summer compared with winter months for all indicator organisms, but not total heterotrophic bacteria.

DISCUSSION

Many communities have constructed centralized systems due to the perception that on-site or decentralized systems are not as advanced technologically, and inferior from a public health and environmental perspective (28). Purchase and management of centralized systems are significantly more expensive than on-site wastewater treatment systems or several individual systems (28). Additionally, low population densities and limited financial resources of rural areas often limit the cost-effective installation of centralized systems. Compared to centralized systems, alternative on-site treatment systems offer great promise for practical and cost-effective wastewater remediation, but also great challenges with respect to regulatory compliance. Nationwide, a significant number of on-site systems have been inadequately designed and/or poorly maintained leading to non-compliance with existing health and environmental regulations (25,28).

The decentralized nature of multiple on-site treatment systems makes conventional water sampling an impractical routine method for community sanitarians to assess ongoing compliance with discharge standards. Telemetric reporting of key performance parameters or alarm situations to a centralized location may eventually offer a more practical way to monitor diverse multiple systems. Currently, few existing on-site systems have been equipped with telemetry equipment. However demonstration projects initiated to investigate the feasibility of telemetry have shown promise (14). Investigation of such experimental systems eventually may lead to an improved understanding of the long-term reliability and function of a variety of innovative, alternative on-site treatment technologies. Questions still to be answered include cost and long-term reliability of telemetry devices installed in independent treatment systems, maintenance requirements and longevity of specific sensing devices, and correlation of telemetric measurement of parameters with conventional indicators for monitoring system performance.

During the current study telemetry was used to report flow, pH, DO, and temperature in an on-site treatment wetland for a three month summer period. Telemetry was found to be most

useful in describing the dynamic hydraulic loading of the treatment system. Consistent with the known occupancy of the multi-user facility, the single day (Sunday) maximum hydraulic load as well as the smaller peak load occurring regularly at mid-week were clearly identified and quantified. These data confirmed Monday mornings as the optimum sampling time to obtain water samples for conventional analyses. Examination of estimated detention times (0.5 to 18.5 days) and dosing volume of the LPP drain field suggest appropriate sizing of the wetland for its intended use. The system is currently in its fourth year of operation and continues to provide effective treatment with no imminent signs of malfunction. Possible statistical correlations between hydraulic loading and parameters such as BOD₅, TSS, and indicator organism enumerations/reductions were examined but significant correlations were not evident.

Monitoring of pH in the septic tank may be useful in tracking insults and/or upsets in the system. Telemetry readings indicated the pH of the first compartment of the system varied ranged from 5.77 to 9.28 during the summer period. High pH values were recorded during a three-week period at the beginning of summer, the remainder of the values ranged from 5 to 7. Heavy chemical input from cleaning products that contain bases can cause fluctuations in the pH of the system. It is possible that the system was disrupted early in the summer do to this sort of activity.

Typically, subsurface flow wetlands are predominately covered by macrophytes and oxygen release from their root system to the rhizosphere has been documented (3,7,8). Monitoring of dissolved oxygen within the wetland system may provide a better understanding the role of plants in these systems. In subsurface flow wetlands, limited contact between the wastewater and the atmosphere and increased biochemical oxygen demand often result in microaerophilic conditions in the system. Bulk oxygenation of the water column is usually ineffective, but localized oxidized environments on or near root surfaces can support growth of aerobic microbes that are thought to promote many treatment processes (9). The wetland in the present study was limited to 40% plant cover, which was concentrated on a single side for aesthetic purposes. Although aeration of wastewater was observed, an increased and uniform plant cover may further improve aeration and result in improved treatment.

Examination revealed significant reductions in TSS, total heterotrophic bacteria, fecal coliforms, enterococci, and *E. coli* during both seasons although reduced efficacy was noticed in winter. Improved treatment by constructed wetlands during summer months compared to winter is not an uncommon phenomenon. Axler et al. (4) reported reduced efficiency of BOD₅ and TSS removal during winter compared to summer in constructed wetlands. Pundsack et al. (22) observed enhanced short-term seasonal removal of *Salmonella* and fecal coliforms in experimental wetlands. Dahab and Surampalli (10), in a study of a constructed wetland, reported a decline in reductions in nutrients and fecal coliforms during winter.

Telemetric monitoring of the temperature may prove to be advantageous in predicting treatment efficacy. Such changes can affect overall efficiency, however, simple correlations between temperature and treatment may not be immediately evident (17,19). Overall the treatment wetland proved to be more effective at reducing standard wastewater parameters and indicator organisms in summer as compared to winter probably as a result of decreased temperature. Telemetric readings used in correlation analysis were recorded during summer months when water and air temperature fluctuations were minimal (18.6 to 25.5°C and 15.1 to 25.7°C, respectively). Telemetric water temperature readings correlated with effluent concentration of fecal coliforms and *E. coli* ($r=0.671, 0.643$) and with BOD₅ reduction ($r=-0.723$). Air temperature correlated with TSS reduction ($r=-0.723$). Recording continues to date and further review of data collected in the upcoming winter will be used to further investigate these correlations.

A unique aspect of the wetland was the high level of treatment that has been maintained for approximately four or five years of operation. Generally, information on longevity of treatment is absent for on-site wastewater treatment systems. The system achieved significant log₁₀ reductions (1.6 to 3.5) for fecal coliforms, enterococci, *E. coli*, and total heterotrophs regardless of season and despite fluctuations in hydraulic loading rate and hydraulic residence time. Reductions in BOD₅ and TSS have been reported that are similar to those seen in this research (64.7 and 89.5 %) and (62.9 and 82.9 %) for winter as compared to summer. Neralla et al. (20) reported an 80-90% reduction in BOD₅, 81% reduction in TSS, and 80-90% reduction in fecal coliforms in a study of eight household constructed wetlands treating

household wastewater. Ottova et al. (21) reported removal rates of 95.3 to 99.9% for fecal coliforms and enterococci removal of 93.2 to 99.5% in five constructed wetland systems. Barrett et al. (5) reported variable log reductions ranging between 0.5 to 4.2 for fecal coliforms and 0.1 to 2.4 for enterococci for several subsurface flow wetlands. Hench et al. (16) reported removal rates in vegetated mesocosms of 42% for BOD₅, 83% for TSS, and >99% removal of fecal coliforms and enterococci, although removal of BOD₅ and TSS in this study were significantly reduced during second year of experiment.

CONCLUSIONS

Real-time monitoring of the constructed wetland proved to be most useful in providing information regarding changes in hydraulic loading rate and retention time of the system. Initial correlations between telemetric recordings and conventional analyses were limited. A longer sampling period with concurrent telemetry is necessary to vigorously investigate possible correlations. Use of telemetry and sampling of the system continues to date and will be further evaluated. Overall the system was able to reduce BOD₅ and significantly reduce TSS, indicator organisms, and total heterotrophs regardless of fluctuations in hydraulic loading rate, retention time, and season. Slight reduction of system efficiency was noticed during winter months, but the wetland appears to meeting most WVDEP regulations. BOD₅ sometimes exceeded the 30 mg L⁻¹ required for direct discharge to a receiving stream, particularly during winter months. However, the wetland discharges to a LPP soil drain field that imparts further treatment. Overall the wetland system appears to function as designed providing reliable and cost-effective on-site wastewater treatment.

Table 1. Telemetric measurement of wetland parameters averaged over differing sampling periods during summer months*

Parameter	Summer	August	7-Day
Hydraulic Loading Rate (L day ⁻¹)	2567 (350)	2575 (580)	2702 (787)
Soil Drainfield Loading Rate (L day ⁻¹)	1284 (222)	1575 (517)	841 (348)
Hydraulic Residence Time (days)	6.6 (5.9)	5.9 (1.1)	3.5 (0.7)
Wetland Temp (°C)	22.5 (0.2)	21.6 (0.2)	22.5 (0.3)
Air Temp (°C)	21.5 (0.3)	21.2 (0.5)	23.8 (0.5)
DO (mg L ⁻¹)	0.94 (0.03)	0.77 (0.05)	1.04 (0.10)
pH	6.80 (0.09)	6.65 (0.03)	6.71 (0.04)

* Mean (standard error in parentheses) of 93 (summer), 31(August), and 7 (7-day) daily means.

Table 2. Physical/chemical parameters and microbial densities of primary clarified wetland influent and wetland effluent over 7-day intensive sampling period*

Parameter	Influent	Effluent	Significance
pH	7.45 (0.08)	7.17 (0.10)	a
Temperature (°C)	20.3 (0.24)	22.7 (0.40)	a
DO (mg L ⁻¹)	2.17 (0.08)	2.45 (0.22)	
Conductivity (mS cm ⁻¹)	1.69 (0.03)	0.81 (0.10)	a
BOD ₅ (mg L ⁻¹)	183.4 (7.2)	14.8 (4.4)	a
TSS (mg L ⁻¹)	31.9 (3.1)	9.2 (1.2)	a
Fecal coliforms (Log ₁₀ CFU 100 mL ⁻¹)	5.1 (0.33)	1.9 (0.02)	a
<i>E. coli</i> (Log ₁₀ CFU 100 mL ⁻¹)	4.2 (0.41)	1.6 (0.00)	a
Enterococci (Log ₁₀ CFU 100 mL ⁻¹)	5.0 (0.17)	2.1 (0.09)	a
Total heterotrophic bacteria (Log ₁₀ CFU mL ⁻¹)	6.8 (0.17)	4.8 (0.15)	a

* Mean (standard error in parentheses) of 7 samples (3 replicates/sample) collected during

^a Indicates effluent was significantly different than influent (P<0.05).

Table 3. Physical and chemical parameters of primary clarified wetland influent and wetland effluent during winter compared to summer*

Parameter	Influent		Effluent		Significance
	Winter	Summer	Winter	Summer	
pH	7.89 (0.26)	7.15 (0.08)	7.13 (0.28)	7.19 (0.05)	a c
Temperature (°C)	8.9 (0.9)	19.4 (0.3)	5.7 (1.4)	22.1 (0.3)	a b c d
DO (mg L ⁻¹)	2.59 (0.65)	1.63 (0.14)	3.97 (0.77)	1.89 (0.15)	a b c
Conductivity (mS cm ⁻¹)	1.38 (0.08)	1.64 (0.02)	1.21 (0.15)	0.83 (0.05)	a b d
BOD ₅ (mg L ⁻¹)	150.8 (32.3)	218.4 (16.0)	82.2 (15.7)	25.5 (5.8)	a d
TSS (mg L ⁻¹)	29.8 (4.4)	37.5 (2.2)	17.6 (2.4)	7.8 (0.8)	b c d

* Mean (standard error in parentheses) of samples (3 replicates/sample) collected during winter (January-March n=6) and summer (June-August n=17).

^a Indicates significant difference between influent winter compared to influent summer (P<0.05).

^b Indicates significant difference between effluent winter compared to effluent summer (P<0.05).

^c Indicates significant difference between influent winter compared to effluent winter (P<0.05).

^d Indicates significant difference between influent summer compared to effluent summer (P<0.05).

Table 4. Microbial densities of primary clarified wetland influent and wetland effluent during winter compared to summer*

Microbial population	Influent		Effluent		Significance
	Winter	Summer	Winter	Summer	
Fecal coliforms (Log_{10} CFU 100 mL^{-1})	5.5 (0.38)	5.6 (0.17)	3.3 (0.19)	2.1 (0.08)	b c d
<i>E. coli</i> (Log_{10} CFU 100 mL^{-1})	5.2 (0.21)	4.7 (0.22)	3.0 (0.18)	2.0 (0.09)	b c d
Enterococci (Log_{10} CFU 100 mL^{-1})	5.5 (0.37)	4.8 (0.17)	3.0 (0.40)	2.2 (0.07)	a b c d
Total heterotrophic bacteria (Log_{10} CFU mL^{-1})	6.9 (0.17)	6.9 (0.10)	5.3 (0.22)	4.9 (0.11)	c d

* Geometric mean (standard error in parentheses) of samples (3 replicates/sample) collected during winter (January-March n=6) and summer (June-August n=17).

^a Indicates significant difference between influent winter compared to influent summer.

^b Indicates significant difference between effluent winter compared to effluent summer.

^c Indicates significant difference between influent winter compared to effluent winter.

^d Indicates significant difference between influent summer compared to effluent summer.

Figure 1. General schematic of the constructed subsurface flow wetland system.

Figure 2. Daily averages for telemetric readings of pH, wetland temperature ($^{\circ}\text{C}$), air temperature ($^{\circ}\text{C}$), and DO (mg L^{-1}) recorded over summer period. Symbols are as follows: ($\circ$) pH; ($\square$) wetland temperature; ($\triangle$) air temperature; ($\diamond$) DO. Missing values for DO are due to probe malfunctions.

Figure 3. Daily averages for calculated hydraulic loading rate (L day^{-1}) and hydraulic retention time (day) recorded over summer period. Symbols are as follows: () hydraulic loading rate; () hydraulic retention time.

Figure 4. Daily averages for telemetric readings of pH, wetland temperature (°C), air temperature (°C), and DO (mg L⁻¹) recorded during August. Symbols are as follows: () pH; () wetland temperature; () air temperature; () DO. Missing values for DO are due to probe malfunctions.

Figure 5. Daily averages for calculated hydraulic loading rate (L day⁻¹) and hydraulic retention time (day) recorded during August. Symbols are as follows: () hydraulic loading rate; () hydraulic retention time.

Figure 6. Daily averages for calculated hydraulic loading rate (L day⁻¹) and hydraulic retention time (day) recorded during 7-day sampling. Symbols are as follows: () hydraulic loading rate; () hydraulic retention time.

Figure 7. Daily averages for conventional analysis of influent and effluent BOD₅ (mg L⁻¹) and TSS (mg L⁻¹) measured over 7-day sampling period. Symbols are as follows: () Influent TSS; () Influent BOD₅; () Effluent TSS; () Effluent BOD₅.

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CHAPTER 2: BACTERIAL DIVERSITY WITHIN A CONSTRUCTED WETLAND TREATING WASTEWATER: A CASE STUDY

INTRODUCTION

Decentralized on-site wastewater treatment systems serve approximately 25% of the U.S. population (40). Individual households most commonly employ a septic tank with a soil drain field, however effective treatment sometimes is not achieved due to poor site conditions such as steep topography, low soil percolation, and limited availability of land. Septic tank effluents frequently are reported as significant sources of groundwater contamination by pathogenic bacteria and viruses (40). Constructed subsurface flow wetlands are now in use both as supplements to and replacements for soil drain fields in on-site wastewater treatment. Primary clarified wastewater effluent enters the wetland and flows horizontally below the surface of the substrate (usually gravel planted with aquatic macrophytes) prior to discharge. The water level within the wetland is maintained constant by an outflow standpipe at the discharge end of the wetland. Subsurface flow wetlands result in decreased mosquito breeding, objectionable odors, and the possibility of human/animal contact with partially treated wastewater. Previous studies have reported acceptable wastewater treatment by subsurface flow wetlands, although seasonal variability and longevity of treatment effectiveness remain a question (4,10,25,31,39).

Physical, chemical, and microbiological processes in subsurface wetlands reduce total suspended solids (TSS), biochemical oxygen demand (BOD_5), as well as the number of pathogenic microorganisms in wastewater (3). Heterotrophic bacteria are thought to play a central role in the wastewater treatment process within constructed subsurface flow wetlands. They dominate both in numbers and biomass, and regulate mineralization and immobilization of organic and inorganic nutrients (1). However, the physiology, survival strategies, and community structure of heterotrophic bacterial populations in constructed subsurface flow wetland systems have not been extensively studied. Relatively little is known about how changes in diversity of the heterotrophic microbial community might affect treatment of

wastewater in these small-scale subsurface flow treatment systems. Heterotrophic bacteria vary in cell size and can be selectively partitioned by membrane filtration. Studies of small or dwarf cells have been conducted in marine environments (19), groundwater (26,37), and soil (12,35). Studies of the small cell fraction of heterotrophic communities have not yet been performed in wastewater wetlands. A better understanding of all fractions of the bacterial community and their dynamics in these systems may eventually contribute to improved system design, functioning, and efficiency of constructed subsurface flow wetland systems.

In a previous study by Hensch et al. (20), changes in functional diversity of wetland heterotrophic bacteria were observed by season and due to the presence or absence of plants. The authors noted failing treatment effectiveness in the experimental wetland system examined and suggested that more detailed studies were called for in robust, well-functioning wetlands. In the present study, heterotrophic bacterial diversity was evaluated in a well-established constructed wetland providing wastewater treatment for a large multi-user facility. Treatment effectiveness and compliance was determined through evaluations of BOD₅, TSS, and enumeration of indicator microbial populations. Community-level physiological profiles (CLPP) of the total cell (TC), large cell (LC), and small cell (SC) fractions of the heterotrophic bacteria community were determined using BIOLOG® ECO plates. In addition, bacterial community diversity was determined using 16S rRNA genes amplified by polymerase chain reaction (PCR) and separated by denaturing gradient gel electrophoresis (DGGE). The objectives were to determine whether passage of primary clarified wastewater through the constructed wetland system resulted in significant reductions of the physical and chemical parameters, fecal indicator bacteria, and total heterotrophic bacteria; and whether the functional and/or genetic diversity of the LC, TC, and SC fractions of the bacterial community varied due to treatment by the wetland system.

METHODS

Wetland System

The system investigated was a constructed subsurface flow gravel wetland (~11.89 m x 5.49 m x 0.45 m) treating primary clarified wastewater from a two-compartment septic tank (Tank 1 5678 L and Tank 2 1893 L) discharging from a multi-user facility (Figure 1). The second compartment of the septic tank acted as a dosing tank for the wetland. The number of users attending the facility varied throughout the week and also throughout the study period; however, the maximum number of users was always on Sunday. It was estimated approximately 1300 people were present on Sundays, with numbers during the remainder of the week ranging from 10-250 depending on on-going programs and functions. The wetland was constructed with 1.3 cm gravel (approximately 44% porosity - based on field testing), lined with a 50 mm flexible liner, and the area surrounding the wetland was constructed with larger stone to limit overland runoff to the wetland. The slope of the wetland was approximately 1% from the inlet to discharge end. The wetland water level was maintained at a constant level of approximately 18 cm. Approximately 40% of the wetland was planted with reed (*Phragmites sp.*) and iris (*Iris sp.*). For aesthetic purposes, one side of the wetland was nearly completely covered with reed and iris plants while the other side contained few plants. Treated effluent flowed by gravity from the wetland to a valve box that led to a holding tank (1893 L). The holding tank dosed a shallow low-pressure pipe (LPP) drain field (5 narrow 0.25 m gravelless pipe trenches) for final disposal. The advantages of this type of drain field include uniform small doses to the entire adsorption area that in turn promotes unsaturated flow and also results in consistent drying/reaeration periods between doses (14). Telemetry was used to report pumping duration from the septic tank to the wetland system. From this data, the average summer hydraulic loading rate from the septic tank to the wetland was calculated to be 2567 L day⁻¹ and the estimated hydraulic retention time within the wetland was 6.6 days. Field porosity of the system was determined to be 44%.

Sampling Methods

A total of 46 grab water samples were collected between 8 and 12 A.M. during January 2004 – August 2004. Influent samples were taken from the second compartment of the septic tank (primary clarified wastewater) and effluent samples were taken from a 15 cm diameter PVC temporary monitoring well installed near the discharge point of the wetland. A total of six intermediate grab samples were collected from six 15 cm diameter PVC temporary monitoring wells installed over the distance of the wetland (Figure 1). Samples were collected in sterile 1-liter Nalgene plastic bottles and packed on ice in an insulated cooler during transportation to the laboratory, where they were analyzed within 24 hours.

Physical/Chemical Analyses

Immediately following sample collection, field measurements of dissolved oxygen, electrical conductivity, pH, and temperature were performed using a YSI Model 556 MPS Meter (Yellow Springs Instruments, Yellow Springs, OH) following standard procedures of the American Public Health Association (2). Upon arrival to the laboratory, samples were refrigerated until processing. At the time of processing, samples were allowed to warm to room temperature prior to analyses. Five-day biochemical oxygen demand (BOD₅) and total suspended solids (TSS) were measured following standard methods (2). Dissolved oxygen values from day five of the BOD₅ incubation were corrected to adjust for any decreases in oxygen of the dilution water blanks over the five-day incubation period.

Bacteriological Analyses

Samples were analyzed for fecal coliforms, *Escherichia coli*, enterococci, and total heterotrophic bacteria by a modified standard method using a Spiral Biotech Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Inc., Norwood, MA) and the standard method membrane filtration technique using 0.45 µm Millipore HA filters (Millipore Corp., Bedford, MA) (2). Serial dilutions were performed or appropriate volumes were filtered to obtain plates with 20-300 colonies (modified standard method) or 20-200 colonies (membrane filtration technique). Plates were incubated for 24 hours at 44.5°C on M-FC medium (Difco, Detroit, MI) for fecal

coliforms, 24 hours at 44.5 °C on EC-MUG for *E. coli* (Difco, Detroit, MI), 48 hours at 35 °C on m-Enterococcus medium for enterococci (Difco, Detroit, MI), and 48 hours at 35 °C on R2A (Difco, Detroit, MI) medium for total heterotrophic bacteria.

Sample Preparation for Culture-Dependent and Culture-Independent Analyses

Bacterial cells from one influent and effluent sample were sequentially collected on 0.45 µm (large cells, LC) and 0.22 µm (small cells, SC) Millipore HA filters (3 filters/sample) (Millipore Corp.). Culture-independent cells (CI) were collected by placing filters into test tubes containing 15 ml of sterile saline solution (0.85% NaCl) and vigorously vortexing for 1-2 min. Culture-dependent cells (CD) were obtained by first placing filters (3 filters/sample) on R2A medium (Difco) and incubating for 48 hours at 35 °C prior to cell collection.

Inoculation and Reading of BIOLOG Plates

An aliquot (9.6 ml), of the CD SC and LC cell suspensions described above, was used to inoculate BIOLOG® ECO plates (100 µl/well, 3 replicates per plate, n=3). Culture-dependent cell suspensions were serially diluted (10^{-2} final dilution). For total cells (TC) CI an aliquot (9.6 ml) of undiluted wastewater was used to inoculate BIOLOG® ECO plates. BIOLOG® plates were incubated in the dark at 25 °C. Plates were read 8 times over approximately 100 hours using a SpectraMAX 340PC plate reader (Molecular Devices Corporation, Sunnyvale, CA) equipped with a 595 nm filter.

Denaturing Gradient Gel Electrophoresis (DGGE)

DNA was extracted from 1.8 ml aliquots of cell suspensions using MoBio Microbial DNA extraction kits (MoBio Laboratories, Carlsbad, CA). The variable V3 region of the 16S rRNA gene from members of the domain *Eubacteria* was PCR amplified by using PRBA338F (5'-ACTCCTACGGGAGGCAGCAG-3') with a GC clamp and PRUN518R (5'-ATTACCGCGGCTGCTGG-3') primers in a Px2 Thermal Cycler (Thermo Electron Co., Needham Heights, MA). Fifty µl PCR reaction mixtures contained 1× PCR buffer (Promega, Madison, WI), 3.2 µmol of MgCl₂ (Promega, Madison, WI), 0.8 µM of deoxynucleoside

triphosphates (Promega, Madison, WI), 2% bovine serum albumin (Fischer Scientific Inc., Hampton, NH), 0.5 μ M (each) of forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 0.5 units of *Taq* polymerase (Promega, Madison, WI), and ~200 ng of template DNA. The PCR protocol included a 5-min initial denaturation at 94°C, 30 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30s, followed by 7 min at 72°C. PCR products were combined, cleaned, and concentrated (100 μ l to 60 μ l) using QIAquick PCR Purification Kits (Qiagen Inc., Valencia, CA). Five μ l of the PCR products were electrophoresed on 1.5% (w/v) agarose gels to check for amplification of a band of the expected size (~180 bp based on *E. coli* positions 338 to 518). Additionally, the quantity of PCR products was analyzed using Quantity One 1-D Analysis Software Version 4.5.1 (Bio-Rad Laboratories, Hercules, CA).

Fifteen μ l (~300 ng) of cleaned PCR product from each sample were loaded on a 8% (w/v) polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) with a 40% - 60% denaturing gradient; where 100% denaturant contains 7 M urea and 40% (v/v) formamide in 0.5X tris-acetic acid-EDTA buffer (TAE). Electrophoresis was performed for 4 minutes at 200 V and 60 °C to drive samples into wells and then 17 hours at 50 V and 60 °C using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Gels were silver-stained using the protocol of Caetano-Anolles and Gresshoff (6) and scanned as tagged image file format (TIFF) files using a HP Scanjet 7400C scanner (Hewlett-Packard Co., Palo Alto, CA).

DGGE Gel Analyses

Bands were detected using the Quantity One 1-D Analysis Software Version 4.5.1 (Bio-Rad Laboratories, Hercules, CA). Lanes were created manually, with anchor points at the top, middle, and bottom of the gel. The relative front calculation was set to follow lane. Background was subtracted from all lanes using a rolling disk with a radius of 15 pixels. Bands were detected manually using a band detection limit (intensity value 0.03) based on visual detection of faint bands on the gel.

Statistical Analyses

All statistical analyses were performed using the SAS Statistical Software Package (SAS Institute Inc., Cary, NC). Differences between physical, chemical, and bacteriological measurements in winter as compared to summer were evaluated using Proc GLM with LSMeans and the tdiff option. Bacteriological data were $\log_{10}$ transformed to obtain geometric means prior to statistical analyses. Unless otherwise noted, significance was based on a P value of less than or equal to 0.05.

For BIOLOG® ECO plates total well color development (TWCD) was calculated as the sum of all blanked absorbance values for the 31 substrate wells. Average well color development (AWCD) was calculated as the mean of all blanked absorbance values for the 31 substrate wells. Substrate diversity (H) was calculated as $H = -\sum p_i \ln p_i$, where p_i is the proportion of total microbial activity on a particular carbon source (ratio of each absorbance value to the TWCD). Substrate richness (S) was calculated as the number of wells with an absorbance value greater than 0.25. Substrate richness per guild was calculated as the number of wells with an absorbance value greater than the AWCD within each guild. Substrate evenness (E) was calculated as $E = H/\ln(S)$, where H is substrate diversity and S is substrate richness. Principal component analysis (PCA) was performed on data normalized by dividing each substrate absorbance value by the AWCD (15,16). This normalizes the data to account for differences in inoculum density of individual BIOLOG® ECO plates. Pearson Product Moment Correlation (Pearson's correlation) was used to evaluate correlations between principal components and AWCD of each substrate.

Total lane intensity (TLI) was calculated as the sum of intensities of detected bands for each lane. Average band intensity (ABI) was calculated as the mean of intensities of detected band for each lane. Substrate diversity (H) was calculated as $H = -\sum p_i \log p_i$, where p_i is the ratio of each detected band intensity to the TLI. Substrate richness (S) was calculated as the number of detected bands within each lane. Substrate evenness (E) was calculated as $E = H/\text{Log}(S)$, where H is substrate diversity and S is substrate richness. PCA was performed on normalized data by dividing each band intensity value by the TLI.

RESULTS AND DISCUSSION

Physical, Chemical, and Bacteriological Analyses

Physical and chemical parameters and microbial densities of wetland influent and wetland effluent are presented in Table 1. Passage of wastewater through the wetland resulted in a significant increase in DO and a significant decrease in pH and conductivity. Overall the system was able to significantly reduce BOD₅ and TSS by 84.6% and 79.7%, respectively. Treatment of the wastewater by the wetland resulted in a 3.2 log reduction of fecal coliforms and a 2.7 log reduction of both *E. coli* and enterococci. Additionally, the system was able to reduce total heterotrophs by 1.9 log. These results indicate a well functioning system, although a slight reduction in overall treatment efficacy was observed during winter months.

Community-Level Physiological Profiles

Community-level physiological profiles (CLPPs) have successfully been used to differentiate microbial communities in activated sludge and other larger scale wastewater treatment systems (8,29,41,42,43). In a study of a municipal wastewater treatment system, Victorio et al. (43) used BIOLOG® to distinguish between heterotrophic microbial populations. Furthermore, the research suggested BIOLOG® might complement conventional testing as a means to detect changes in the microbial population due to factors such as temperature, ionic strength, effluent concentration, and inadvertent upsets, and also to measure recovery periods after system upsets. CLPPs generated by BIOLOG® rarely have been used to investigate constructed wetlands (20). Further investigations of heterotrophic bacteria in constructed wetlands with BIOLOG® may help to understand their role in the wastewater treatment process within these systems.

In the present study CLPP analyses were performed using BIOLOG® ECO plates to determine how wastewater passage through the wetland affected microbial functional diversity. Garland and Mills (15) first suggested the use of BIOLOG microtitre plates to generate community level physiological profiles (CLPPs) from the number and types of carbon

substrates used by the heterotrophic bacterial community. Limitations to the CLPP approach have been extensively scrutinized and discussed (17,22,34). The assay most probably characterizes only fast-growing, metabolically active, copiotrophic bacteria, and the environmental relevance of the carbon utilization profile obtained is questionable. However, the assay is relatively inexpensive, convenient, reproducible, and allows rapid indirect characterization of changes in microbial community structure. Studies suggest that changes in CLPP profiles indirectly reflect actual changes in the underlying microbial community (38,5). The assay is complementary to molecular techniques for polyphasic community characterizations (9,11,18,21).

CLPPs are potentially sensitive to large differences in initial inoculum density, since color development depends on rate of growth in the substrate wells. We used undiluted wastewater as a direct inoculum in the culture-independent approach to investigate spatial trends as wastewater passed through the wetland from influent to effluent. Other samples were sequentially filtered through 0.45 then 0.22 μ m filters to fractionate the community by size for comparison of large (LC) and small (SC) cells. These filters were incubated on R2A medium for 48 hours prior to collection of cells (culture-dependent analyses). The culture-dependent approach allowed cell resuscitation and provided growth of a sufficient inoculum density from the low numbers of small cells originally present in effluent samples, allowing appropriate kinetics of color development for CLPP (Figure 2). Typically, BIOLOG® data are compared after a fixed incubation time when absorbance values for most substrates are increasing, but have not approached saturation (16). After inspection, data obtained at 54.5 hours for the LC and SC fractions (culture-dependent) and 75.5 hours for the TC fractions (culture-independent) were chosen for further analysis (Figure 2).

Calculated CLPP indices are presented in Table 2. In order to eliminate weak responses from the calculations, an average absorbance value greater than 0.25 was required for a substrate to be positively utilized (17). TWCD, AWCD, and evenness of TC, LC and SC generally were higher in effluent compared to influent samples (Table 2). These data demonstrate the presence of an active microbial population in the effluent. Richness was high for all samples (26-30 substrates utilized) with the exception of TC, well # 7 (22 substrates utilized, Table 2).

The diversity indices calculated for TC and LC in influent and effluent samples were similar (3.14, 3.28, 3.26 and 3.35, respectively). Diversity indices calculated for influent SC (3.13) and effluent SC (3.30) were significantly different (Table 2). These data contrast those of Hench et al. (20) who used BIOLOG® GN plates, which contain 95 carbon substrates, and observed decreases in AWCD, richness, and diversity between influent and effluent of microcosm wetlands treating wastewater.

Diversity indices provide no information about the classes of substrates preferentially utilized by bacterial communities. BIOLOG® ECO plates were originally designed for environmental studies and contain 31 carbon substrates relevant to growth of natural microbial communities (7). Therefore, it is not uncommon for many of these individual substrates to be positively utilized in an inoculated plate. Vahjen et al. (44) have defined a strongly utilized carbon substrate as one for which the absorbance was greater than the AWCD. Dobranic and Zak (13) divided the carbon substrates in BIOLOG® ECO plates into substrate guilds. Combining these concepts, we examined average richness within substrate guilds for strongly utilized substrates (Table 3). One clear difference observed was the significant increase in utilization of carboxylic acids in effluent compared to influent LC and SC, but not TC fractions. In contrast amines/amides were not strongly utilized by any fraction of the bacterial community examined. Carbohydrate, carboxylic acid, and polymer utilization generally increased in effluent LC compared to influent LC. Carboxylic acid and amino acid utilization was higher in effluent SC compared to influent SC. Utilization by the TC fraction increased for polymers and amines/amides, and decreased for amino acids when influent was compared to effluent.

Principal component analysis (PCA) using normalized AWCD values for the LC and SC fractions of the bacterial communities is presented in Figure 3. Analysis showed principal component (PC) 1 accounted for 50.7% while PC2 accounted for 21.7% of the observed total variation. PC 1 was most positively correlated with α -ketobutyric acid ($r = 0.9650$) and most negatively correlated with α -D-lactose ($r = -0.8698$). PC 2 was most positively correlated with D-cellobiose ($r = 0.8327$) and most negatively correlated with L-asparagine ($r = -0.7034$). The two-dimensional plot of PC 1 and PC 2 separated the samples into two distinct groupings

based primarily on position in the wetland, but not cell size (Figure 3). Influent communities differed from effluent communities, and SC and LC in each location exhibited similar substrate utilization patterns.

Principal component analysis (PCA) using normalized AWCD values for the TC fraction of the bacterial communities is presented in Figure 4. Compared with data presented in Figure 3, less of the total variation was accounted for in PC1 (29.2%) and PC2 (17.9%). PC 1 was most positively correlated with phenylethyl-amine ($r = 0.8720$) and was negatively correlated D-cellobiose ($r = -0.8936$). PC 2 was most positively correlated with D-glucosaminic acid ($r = 0.9091$) and negatively correlated with glycogen ($r = -0.6654$). The two-dimensional plot of PC 1 and PC 2 generally separated the samples according to location within the wetland. The data suggest that the TC heterotrophic community of the influent was distinctly different than that of the effluent and that the TC heterotrophic community gradually changed during passage of the wastewater through the wetland.

Denaturing Gradient Gel Electrophoresis Analyses

DGGE has been used in several environments including soil (18,27,30), surface water (36), and wastewater (24) and has proven to be a relatively simple approach to obtain profiles of microbial populations. These DGGE profiles can be linked to temporal or spatial differences in population structure that occur in response to environmental factors. Research using DGGE in wastewater has thus far been focused on pharmaceutical and metallurgic wastewater (24,32), and activated sludge systems (23,33).

Inocula used in CLPP analyses also were used for genetic examination of bacterial diversity using DGGE fingerprints generated from PCR amplification of community 16S rRNA genes. The DGGE profiles of the bacterial communities for LC and SC fractions using both culture-dependent and culture-independent techniques are presented in Figure 5. DGGE analysis resulted in clear discernable banding patterns from both culture-independent and culture-dependent samples. Major bands with intensities greater than 0.03 were identified as distinct operational taxonomic units (OTUs) and used in comparative analyses. Total OTUs (richness,

Table 4) ranged from 17 to 37 among all samples. Only two OTUs were shared commonly among all samples. Five OTUs were shared among all culture-dependent analyses, and 8 OTUs were shared commonly among culture-independent analyses. Among all size classes and culturing methods, influent and effluent samples shared a number of common bands, ranging from 15 to 24 OTUs. The greatest difference between influent and effluent OTUs was 10, observed in the SC culture-dependent analyses. Bacterial diversity indices calculated for all samples are presented in Table 4. Bacterial diversity increased slightly in effluent LC compared to influent LC and decreased in effluent SC compared to influent SC using culture-dependent analysis. Conversely, using culture-independent analysis diversity of effluent LC decreased and effluent SC increased compared to influent.

Unweighted UPGMA (based on presence or absence of bands) and weighted UPGMA (based on presence or absence of bands and band intensity) dendrograms constructed using Dice similarity matrix data for the PCR-amplified 16S rRNA genes resulted in different clustering patterns (Figure 6). The unweighted UPGMA dendrogram separated the data generally according to cell size into five distinct clusters (Figure 6a). When band intensity also was taken into consideration data separated into four distinct clusters according to cell size (LC or SC) and analysis method (CD or CI, Figure 6b). PCA analysis performed using absence or presence and normalized intensity values for each detected band showed separation similar to the weighted UPGMA dendrogram (Figure 7).

It is interesting, but perhaps not surprising, that CLPP using BIOLOG® and DGGE analyses resulted in contrasting views concerning the distribution of heterotrophic bacterial diversity in the wastewater wetland. CLPP is a measure of potential physiological diversity, not actual catabolic activity (34). Furthermore, CLPP is a selective enrichment since positive color development in the wells of BIOLOG® plates can be caused by active respiration and growth of a limited number of organisms. Much like CLPP using BIOLOG®, limitations of DGEE should also be considered when interpreting results. DGGE provides an estimate of actual genetic diversity of the dominant members of microbial communities, but also is subject to limitations such as the method of DNA extraction and PCR bias (28) and reveals nothing

about the activity of microbial populations. Typically, cells must constitute at least 1% of the total microbial population to provide a visible band on DGGE gels (30).

CONCLUSIONS

A research approach incorporating both traditional methodology and PCR-based molecular techniques was used to examine changes in heterotrophic community structure in wastewater treated by a constructed subsurface flow wetland. In this study, PCA of CLPPs grouped samples according to location in the wetland system suggesting redundant physiologies among the LC and SC fractions in the influent and effluent, but distinct potential functional diversity at each location (culture-dependent analysis). These data are consistent with CLPP patterns generated using the TC fraction that revealed a gradient of physiological potential from wetland influent to effluent. In contrast, PCA of DGGE profiles for the LC and SC fractions grouped according to cell size, rather than sample location. These data suggest that size fractionation resulted in distinct populations and that DNA from both populations could be recovered from samples throughout the wetland. However, simply because DNA was recovered it cannot be inferred that all members of these communities are viable and active throughout the wetland. Indeed, microbial enumerations in this research suggest significant reductions in viable bacteria due to passage through the wetland.

In future studies it may be interesting to couple reverse transcription PCR (RT-PCR) of rRNA with DGGE to better assess dominant active populations throughout the wetland. The working hypothesis would be that PCA generated from these methods would be more similar to those of CLPP, and would separate populations by both cell size and location. Further research should be conducted to evaluate the effect of environmental factors on CLPPs and DGGE profiles of the bacterial communities present in full-scale and on-site wastewater treatment systems.

Table 1. Physical and chemical parameters and microbial densities of primary clarified wetland influent and wetland effluent over entire sampling period*

Parameter	Influent	Effluent	Percent removal/ Log10 reduction
pH	7.35 (0.11)	7.17 (0.08) ^a	
Temperature (°C)	16.5 (1.1)	17.6 (1.6)	
DO (mg L ⁻¹)	1.82 (0.18)	2.30 (0.26) ^a	
Conductivity (mS cm ⁻¹)	1.57 (0.03)	0.93 (0.06) ^a	
BOD ₅ (mg L ⁻¹)	206.5 (15.3)	37.7 (8.4) ^a	84.6
TSS (mg L ⁻¹)	36.6 (2.0)	9.3 (1.1) ^a	79.7
Fecal coliforms (Log ₁₀ CFU 100 mL ⁻¹)	5.6 (0.16)	2.4 (0.13) ^a	3.2
<i>E. coli</i> (Log ₁₀ CFU 100 mL ⁻¹)	4.8 (0.19)	2.1 (0.12) ^a	2.7
Enterococci (Log ₁₀ CFU 100 mL ⁻¹)	5.0 (0.17)	2.3 (0.12) ^a	2.7
Total heterotrophic bacteria (Log ₁₀ CFU mL ⁻¹)	6.9 (0.09)	5.0 (0.10) ^a	1.9

* Mean (standard error in parentheses) of samples (n=23) (3 replicates/sample) collected over entire sampling period.

^a Indicates effluent was significantly different than influent (P<0.05).

Table 2. Bacterial functional diversity indices as determined with BIOLOG® ECO plates

Cell Fraction	Sample	TWCD ^{a,f}	AWCD ^{b,f}	Richness ^{c,f}	Diversity ^{d,f}	Evenness ^{e,f}
<u>Culture Dependent Analysis</u>						
Large Cells	Influent	32.8 b	1.06 b	30 a	3.28 a	0.96 b
	Effluent	43.3 a	1.40 a	30 a	3.35 a	0.98 a
Small Cells	Influent	32.9 b	1.06 b	27 b	3.13 b	0.96 b
	Effluent	41.9 a	1.35 a	29 a b	3.30 a	0.98 a
<u>Culture Independent Analysis</u>						
Total Cells	Influent	36.7 b	1.18 b	26 c	3.14 c	0.96 c
	Well 2	40.4 ab	1.30 ab	30 a	3.34 a	0.99 a
	Well 3	42.3 a	1.36 a	28 abc	3.22 bc	0.97 bc
	Well 4	41.0 ab	1.32 ab	29 ab	3.32 ab	0.99 a
	Well 5	39.7 ab	1.28 ab	27 bc	3.24 abc	0.98 ab
	Well 6	44.1 a	1.42 a	29 ab	3.27 ab	0.97 abc
	Well 7	27.5 c	0.89 c	22 d	2.88 d	0.94 d
	Effluent	41.0 ab	1.32 ab	28 ab	3.26 abc	0.97 abc

^a Total well color development (TWCD) = mean sum of blanked absorbance values from triplicate analysis.

^b Average well color development (AWCD) = TWCD/31.

^c Richness = mean number of wells greater than 0.25 from triplicate analysis.

^d Diversity = $H = -\sum (p_i \cdot \ln p_i)$, where p_i = mean of each absorbance value / TWCD.

^e Evenness = $E = H / \ln(S)$, where H is diversity and S is richness.

^f Mean values in each column within culture-dependent and within culture-independent analysis followed by the same letter are not significantly different ($P < 0.05$) as determined by Tukey's Studentized Range (HSD) Test.

Table 3. Richness within substrate guilds as determined by strongly utilized substrates with BIOLOG® ECO plates

Cell Fraction	Sample	Carbohydrates ^a	Carboxylic Acids ^a	Polymers ^a	Amino Acids ^a	Amines/Amides ^a	Misc. ^a
<u>Culture Dependent Analysis</u>							
Large Cells	Influent	4 b	4 b	2 b	2 a	0 a	1 a
	Effluent	6 ab	6 a	4 ab	2 a	0 a	1 a
Small Cells	Influent	5 ab	4 b	3 ab	2 a	0 a	2 a
	Effluent	5 a	6 a	3 ab	3 a	0 a	1 a
<u>Culture Independent Analysis</u>							
Total Cells	Influent	6 a	4 ab	3 ab	2 a	0 c	1 a
	Well 2	6 a	5 ab	3 b	2 a	0 c	1 a
	Well 3	6 a	5 ab	3 ab	3 a	0 c	1 a
	Well 4	6 a	6 a	3 ab	2 a	2 ab	1 a
	Well 5	6 a	6 a	3 ab	3 a	2 a	1 a
	Well 6	6 a	4 ab	3 ab	3 a	1 ab	2 a
	Well 7	5 a	2 b	3 b	1 a	1 bc	2 a
	Effluent	6 a	4 ab	4 a	3 a	1 abc	1 a

^a Mean values in each column within culture-dependent and within culture-independent analysis followed by the same letter are not significantly different ($P < 0.05$) as determined by Tukey's Studentized Range (HSD) Test.

Table 4. Bacterial diversity indices as determined with DGGE profiles

Cell Fraction	Sample	TLI ^a	ABI ^b	Richness ^c	Diversity ^d	Evenness ^e
Culture Dependent Analysis						
Large Cells	Influent	2.99	0.11	28	1.37	0.95
	Effluent	5.47	0.19	29	1.40	0.96
Small Cells	Influent	5.75	0.16	37	1.48	0.94
	Effluent	4.10	0.24	17	1.16	0.94
Culture Independent Analysis						
Large Cells	Influent	5.97	0.21	28	1.38	0.95
	Effluent	5.18	0.21	25	1.32	0.95
Small Cells	Influent	3.05	0.15	20	1.22	0.94
	Effluent	2.79	0.12	23	1.26	0.93

^a Total lane intensity (TLI) = sum of normalized intensity values from detected bands.

^b Average band intensity (ABI) = TLI/number of detected bands.

^c Richness = number of detected bands (intensity greater than 0.03).

^d Diversity = $H = -\sum (p_i \cdot \log p_i)$, where p_i = each detected band intensity/TLI.

^e Evenness = $E = H/\ln(S)$, where H is diversity and S is richness.

Figure 1. General schematic of the constructed subsurface flow wetland system

Figure 2. Average well color development (AWCD) from BIOLOG® ECO plates for the total cell (TC) fraction using culture-independent (CI) analysis and the large cell (LC) and small cell (SC) fractions using culture-dependent analysis. Symbols are as follows: (○) Influent TC CI; (□) Effluent TC CI; (△) Influent LC CD; (■) Effluent LC CD; (◇) Influent SC CD; (×) Effluent SC CD. (Note: Some symbols are obscured by symbols at several points on the figure.)

Figure 3. Principal component analysis based on normalized AWCD (average absorbance value for each substrate divided by AWCD) from BIOLOG® ECO plates. Values in parenthesis indicate the proportion of the total variation accounted for by each principal component. Symbols are as follows: (■) Influent LC CD; (◻) Influent SC CD; (|) Effluent LC CD; (○) Effluent SC CD.

Figure 4. Principal component analysis based on normalized AWCD (average absorbance value for each substrate divided by AWCD) from BIOLOG® ECO plates for the total cell (TC) fraction using culture-independent (CI) analysis. Values in parenthesis indicate the proportion of the total variation accounted for by each principal component. Symbols are as follows: () Influent TC CI; () Well 2 TC CD; () Well 3 TC CD; () Well 4 TC CD; () Well 5 TC CD; () Well 6 TC CD; () Well 7 TC CD; () Effluent TC CI.

Figure 5. DGGE analysis of PCR-amplified 16S rRNA genes from the LC and SC fractions of the wetland bacterial communities. The figure shows profiles of Influent LC CD (lane 1); Influent SC CD (lane 2); Effluent LC CD (lane 3); Effluent SC CD (lane 4); Influent LC CI (lane 6); Influent SC CI (lane 7); Effluent LC CI (lane 8); Effluent SC CI (lane 9).

Figure 6. Unweighted (a) UPGMA and weighted (b) UPGMA dendrograms constructed using dice similarity matrix generated from DGGE profiles of PCR-amplified 16S rRNA genes from the LC and SC fractions of the wetland bacterial communities. Lane numbers represent the following samples: Influent LC CD (lane 1); Influent SC CD (lane 2); Effluent LC CD (lane 3); Effluent SC CD (lane 4); Influent LC CI (lane 6); Influent SC CI (lane 7); Effluent LC CI (lane 8); Effluent SC CI (lane 9).

Figure 7. Principal component analysis based on normalized band intensity (band intensity value for each band divided by total lane intensity) from DGGE analysis of PCR-amplified 16S rRNA genes. Values in parenthesis indicate the proportion of the total variation accounted for by each principal component. Symbols are as follows: () Influent LC CD; () Effluent LC CD; () Influent SC CD; () Effluent SC CD; () Influent LC CI; () Effluent LC CI; () Influent SC CI; () Effluent SC CI.

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Appendix 1. Photographs of Constructed Subsurface Flow Wetland Research Site

Figure 1. Study Site – Constructed subsurface flow gravel wetland treating wastewater from a multi-user facility.

Figure 2. 15 cm diameter PVC temporary monitoring well installed in wetland used for sample collection. Note electrical wiring leading into well for water temperature probe located in well.

Figure 3. Inside of temporary monitoring well and view of temperature probe inside well.

Figure 4. View of lids to two-compartment septic tank and telemetry panel. View of inside of first compartment of septic tank (top right).

Figure 5. View of wetland system from the discharge end. Lid of pump tank chamber (doses to the soil drain field) in the foreground.

Figure 6. View of inside of discharge box located at discharge end of wetland. Elbow shaped standpipe regulates water level of wetland. Two oxygen probes are installed in the standpipe.

Figure 7. Inside of telemetry panel. Zoomed in view of DO meters (middle) and Sensaphone® 2000 (right) which is used to download telemetry readings via a telephone line to a computer at WVU.