Transactions of the Illinois State Academy of Science
(2003), Volume 96, #2, pp. 87-98

received 2/5/02
accepted 3/29/03

Identification of Diverse Wetland Microbial Communities and Populations Using PLFA and PCR-DGGE Analysis Techniques

Timothy R. Kelley[1], Ph.D and Audrey E. Hentzen Ph.D., MT (ASCP)[2]
[1]corresponding author
Associate Professor, Environmental Health Program
5220 Health Sciences Dept., Illinois State University (ISU), Normal, IL 61790-5220
Tel.# (309) 438-5142, Fax.#(309) 438-2450
[2]Director, Phlebotomy Program
Division of Health and Life Sciences
Casper College, 125 College Drive, Casper, WY 82609

ABSTRACT

Phospholipid fatty acid (PLFA) and 16S ribosomal DNA polymerase chain reaction amplification-denaturing gradient gel electrophoresis (PCR-DGGE) techniques were used to screen water samples collected from an aquatic wetland system currently undergoing restoration for microbial communities and identify predominant bacterial populations. The approximately 2,000-acre "Spunky Bottoms" land tract, purchased by The Nature Conservancy, is currently being restored with The Wetlands Initiative to conditions before the area was leveed from the Illinois River and drained for intensive agriculture (circa 1900). Representative water samples of approximately one liter were aseptically collected and analyzed for microbial communities and bacterial populations. Samples were analyzed by gas chromatography/mass spectrometry to determine PLFA profiles for each water sample. Comparison of PLFA profiles of all samples analyzed indicated primarily eukaryotic organisms, including fungi, protozoa, and diatoms. Biomarkers for Gram-negative bacteria were also detected in all samples analyzed while low proportions of biomarkers for Gram-positive bacteria were observed. Biomass content (total PLFA/sample) was approximately one fold higher in water samples collected from two of seven locations. Predominant PCR-DGGE DNA fragments were selected, excised, sequenced, and predominant microbial species were characterized based upon sequence homology to previously identified sequences contained in the National Center for Biotechnology and Ribosomal Data Project databases. Results identified diverse microbial communities including microorganisms that may substantially contribute to biogeochemical cycling of elements, including N and P. Sequence analysis of DNA fragments showed similarity indices of 0.8 to 1.0 to α-Proteobacteria, Flavobacterium, Flexibacter-Cytophaga-Bacteroides group, Gram-positive and Gram-negative bacteria, Cyanobacteria and Chloroplasts. Determination of predominant microbial communities and populations

may be used to provide a basis of comparison for future research and evaluate potential contributions to biogeochemical elemental cycling.

INTRODUCTION

Natural aquatic wetlands protect water quality by serving as a buffer system to slow water runoff from storm events and allow infiltration into soils, percolation into soil-groundwater systems, and allow time for water purification through natural physical, chemical, and biological processes (Hey and Philippi, 1999; Hammer, 1997; Kadlec and Knight, 1996; National Research Council, 1995; Whigham, et al. 1993). Wetland restoration has been proposed as a means of reducing nitrogen (N) transport to coastal waters (Fleischer and Stibe, 1991). Both point and non-point sources of potential wetland water pollution exist due to location of adjacent residential and agricultural land use (USEPA, 1995; IEPA, 1994, 1995).

The approximately 2,000-acre Spunky Bottoms wetland site in Southern Illinois is owned by The Nature Conservancy and is currently undergoing restoration to more natural conditions which existed prior to leveeing and agricultural use (circa 1900). These efforts are in cooperation with The Wetlands Initiative (TWI), funded by a grant from The National Fish and Wildlife Foundation (NFWF). Restoration goals include increasing the water level and planting native grasses and other wetland flora to provide habitats for aquatic organisms. Water flow through Spunky Bottoms wetland is primarily from northwest to southeast, entering the wetland through runoff from the upland topography to the west. Water then flows from the wetland through drainage ditches (North market, main road, and south Cox) to the pumphouse. Previously, excess water collected was pumped over the levy into the Illinois River to facilitate agricultural land use (Figure 1). This approach continues to be used to control water levels in the wetland during restoration efforts.

New biochemical and molecular biology techniques have been developed which identify microbial organisms in aquatic systems. Phospholipid fatty acid (PLFA) analysis is a technique by which fatty acids are extracted from microorganisms, characterized, and ratios compared to known ratios of fatty acids present in cell membranes and other microbial structures (Stephen et al., 1999a,b). Databases of phospholipid fatty acid profiles have been generated for a variety of microorganisms to facilitate their identification. PLFA analysis has been used to examine microbial communities and populations and their changes for a variety of marine, aquatic, and terrestrial systems. Additionally, polymerase chain reaction amplification-denaturing gradient gel electrophoresis (PCR-DGGE) analysis has been used to isolate microbial DNA and thereby identify predominant microbial populations in a variety of systems. The PCR is a technique used to geometrically increase concentrations of specific DNA fragments to provide enough genetic material for identification by techniques such as gel electrophoresis. Denaturing gradient gel electrophoresis separates and distinguishes DNA fragments by establishing a denaturing gradient that separates DNA into discrete bands that can then be excised and sequenced for identification. A variety of efforts, including those using molecular biology techniques, have been used to illustrate the diversity of microorganisms in marine (Crump, et al., 1999, 1996; Gonzalez and Moran, 1997; Zwart, et al., 1998a,b), estuarine

(Crump, et al. , 1999; 1996), and aquatic (Methe, et al. 1998; Hiorns, et al., 1997) environments. Efforts have also been made to compare marine and freshwater bacterial communities (Mullins, et al., 1995). The work of Duncan and Groffman (1994) used biochemical techniques to compare microbial parameters in natural and constructed wetlands. Their investigation focused on determination of microbial biomass, soil respiration, denitrification enzyme activity, and potential net nitrogen mineralization rather than identification of potential contributors to restoration microbial populations and communities. Limited data on wetland microbial communities, populations and their potential to contribute to biogeochemical cycling of elements is available.

The focus of this investigation is the biochemical and molecular profiling of wetland communities with the identification of organisms that potentially contribute to wetland recovery and restoration. Water samples were collected from Spunky Bottoms wetland and analyzed using PLFA and PCR-DGGE techniques. This provided information concerning predominant microbial communities, populations and in some cases, genera identification. The data generated by this study may be used as a basis for comparison of studies and potentially, to evaluate the role(s) of certain microorganisms in biogeochemical cycling of N, P, and other elements in the wetland.

MATERIALS AND METHODS

Seven sites within and adjacent to the wetland were identified by The Wetlands Initiative (TWI) as representative of geographical distribution of surface water flow throughout the area. Water samples of approximately 1.0-L were collected aseptically from four sites (North Cox, South Market, Main Road and Pump House) on July 13, 2000 and shipped overnight in insulated coolers for analysis. Water samples were collected from three additional sites (Middle Creek, Snyder's Landing, and the Illinois River) and one duplicate site (Pump House) on November 13, 2000 and also shipped overnight for analysis.

Phospholipid Fatty Acid (PLFA) Analyses

Lipids were recovered using the modified Bligh and Dyer method (White, et al., 1997). Lipid extractions were performed using one-phase chloroform-methanol-buffer extractant. Lipids were recovered, dissolved in chloroform, and fractionated on disposable silicic acid columns into neutral-, glyco-, and polar-lipid fractions. The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane. PLFA were analyzed by gas chromatography with peak confirmation performed by electron impact mass spectrometry (GC/MS). PLFA nomenclature follows the pattern of A:BωC. The "A" position identifies the total number of carbon atoms in the fatty acid. Position B is the number of double bonds from the aliphatic (ω) end of the molecule. Position "C" designates the carbon atom from the aliphatic end before the double bond. This is followed by a "c" for cis or a "t" for trans configuration. The prefix "i" and "a" stand for iso and anteiso branching. Midchain branching is noted by "me", and cyclopropyl fatty acids are designated as "cy" (White, et al., 1997). Example: 18:1ω7c is 18 carbons long with one double bond occurring at the 7th carbon atom from the ω end, and the hydrogen molecules attached to the doubly bonded carbon molecules are in the cis conformation (Table 3).

Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) Analyses

Nucleic acid extraction was performed using a bead-beating system (Stephen, et al., 1999a,b) to disrupt cell structure. Sodium phosphate buffer, chaotropic reagent, glass beads, and the sample were agitated in a microcentrifuge tube using a high speed bead beater. Chloroform was added, mixed thoroughly, and the tube was recentrifuged. The aqueous supernatant was collected and phenol/cholroform/isoamyl alcohol (24:24:1) extracted. Glycogen was added and the DNA was precipitated from the aqueous phase with an equal volume of isopropanol. DNA was pelleted by centrifugation, washed with 80% ethanol, air-dried, and re-dissolved in Tris buffer, pH 8.0. The DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer. PCR amplification of 16S rDNA gene fragments was performed as described in Muyzer et al. (1993) with modifications as described. Thermocycling consisted of 35 cycles of 92°C for 45 sec, 55°C for 30 sec, and 68°C for 45 sec, using 1.25 units of Expand High Fidelity polymerase and 10 pmole each primer (forward and reverse, with the forward primer containing a 40 bp GC-clamp) in a total volume of 25 μL. Thermocycling was performed using a "Robocycler™" PCR block. The primers targeted DNA encoding eubacterial 16S rRNA regions corresponding to *E. coli* positions 341-534. A portion (20%) of each PCR reaction product was analyzed by agarose gel electrophoresis (1.5% agarose, 1x TAE buffer) and ethidium bromide fluorescence. The amount of DNA used for DGGE was standardized to 600 ng by comparison to molecular weight standards using Alpha Imager™ software. DGGE employed a D-Code 16/16 cm gel system maintained at a constant temperature of 60°C in 6L of 0.5 x TAE buffer (20mM Tris actate, 0.5mM EDTA, pH 8.0). Denaturing gradients were formed between 30 – 65 % denaturant (with 100% denaturant defined as 7 M urea, 40% v/v formamide). Gels were electrophoresed at 35V for 16 hr. Gels were stained with ethidium bromide (0.5 mg/L) and destained twice in 0.5 x TAE for 15 min. each. Gel images were captured using an Alpha Imager™ system . The central 1-mm portion of strong DGGE bands were excised using a razor blade and soaked in 50-μL of purified water overnight. A portion (15-μL) was used as the template in a PCR sequencing reaction under conditions as described above. Sequence reaction products were purified by electrophoresis through a 1.2% agarose/TAE gel followed by glassmilk extraction (Gene-Clean™ kit). Purified DNA was sequenced with an ABI-Prism automatic sequencer model 377 with dye terminators. Sequence comparison and identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

RESULTS AND DISCUSSION

PLFA Analyses

Results of the first set of analyses (July 2000) indicated that biomass content was highest in the Main Road Ditch sample (1368 pmole/mL), with the second highest biomass observed in the Snyder Landing sample (254 pmole/mL) (Figure 2a,b and Table 1and 2). Results of the first set of analyses (July, 2000) also indicated that the relative proportion of Gram-negative biomarkers was lowest in the Main Road Ditch sample and highest in the North Market Ditch sample (Table 1 and 2). Biomarkers for Gram-negative bacteria were detected in all seven samples, confirming previous results of indicator bacteria

analyses generated from these sites (Kelley and Huddleston, 2001). Low proportions of biomarkers prominent in Gram-positive bacteria were detected in all seven samples. Results of the second set of analyses (November, 2000) indicated that biomass content was lowest in the Middle Creek sample and highest in the Snyder's landing sample (Table 1 and 2). Ratios of cyclopropyl to cis fatty acids (cy/ω7c) show whether Gram-negative communities in these samples are in log phase of growth or not. Very fast turnover rates have ratios that are high. Results of the second set of analyses (November, 2000) indicated that the turnover rate of the Gram-negative community was fastest in the Middle Creek sample (Table 3).

Phospholipid fatty acids (PLFA) found within the membranes of all living cells decompose quickly upon cell death. Structural groups are defined according to PLFA molecular structure as related to fatty acid biosynthesis. Normal saturate fatty acids are ubiquitous while terminally branched saturates (TerBrSats) are attributed to Gram-positive bacteria and some anaerobic Gram-negatives. Branched monoenoic are found in anaerobic metal reducing bacteria and mid chain branched are common in metal reducers and aerobic Actinomycetes. Monoenoic are in Gram-negative bacteria and polyenoics are found in eukaryotic organisms (White et al., 1997). Bacterial biomass is calculated based upon PLFA attributed specifically to prokaryotes whereas eukaryotic biomass is based on PLFA associated with higher organisms. PLFA profile comparisons indicated that all water samples analyzed contained larger quantities of eukaryotic organisms (Figure 2a,b and Table 2) compare to prokaryotic. In particular, eukaryotic biomarkers found in fungi (18:2ω6), protozoa (20:4ω6) and diatoms (20:5ω3) were detected in all seven samples (Table 2).

PCR-DGGE Analyses

DNA profiles revealed diverse bacterial communities, of which the most prominent members were excised and sequenced. Gel images are shown in Fig. 4a,b. DNA profiles showed very diverse bacterial banding patterns that were similar for each of the seven sampling locations. Banding patterns and relative intensities of the recovered bands provide a measure of change in the community. Species must constitute at least 1-2% of the total bacterial community to form a visible band after PCR amplification. Dominant bands (labeled A-G and A-J) were excised, sequenced, and identified (Figure 4a,b). Identifications were based upon the DNA sequence similarity to known sequence found in the Ribosomal Database Project (RDP) database. Similarity indices above 0.800 are considered excellent, 0.600-0.700 are good and below 0.500 are considered to be unique sequences. Note that identification (even phylogenetic affiliation) of bands C, D, and F (Table 4a., 7/00); and D and J (Table 4b., 11/00) failed due to inadequate sequence information and that many of the bands generated on 7/00 (Table 4a.) remained unclassified based upon their comparison to the RDP. Further characterization of the these sequences through blast search comparison to the National Center for Biotechnology data base revealed more information for some of the unclassified bands (data not shown). Clone CR-FL20 was characterized as alpha-proteobacterium species. Clones CR-FL14, CR-FL-FL3, CRE-FL13 and LCK-79 were most similar to Actinomycete species.

CONCLUSIONS

Results generated from PLFA and PCR-DGGE analyses of wetland water samples indicate diverse microbial communities including fungi, algae, protozoa, diatoms, and bacteria; similar to previous research generated on aquatic microbial communities. Biomarkers for Gram-negative bacteria were more abundant than Gram-positive bacteria, in accordance with previous research on this wetland to determine indicator bacteria concentrations (Kelley and Huddleston, 2001). Predominant microorganisms identified by PCR-DGGE include *Flavobacterium* spp., alpha-proteobacteria, *Actinomycete* spp., *Prochloroccus* spp. as well as several unclassified clones. These organisms may contribute significantly to biogeochemical cycling of elements such as N, P, and C. Alpha-proteobacterium contain organisms including the Purple Nonsulfur Bacteria, the *Rhizobiaceae* Family which contains the genera *Rhizobium, Agrobacterium* and nitrifying bacteria *Bradyrhizobiaceae – Nitrobacter* and *Nitrococcus*. Purple Nonsulfur Bacteria are capable of anoxygenic photosynthesis utilizing a variety of energy sources for metabolism making then photoorganoheterotrophs. Additionally, *Bradyrhizpbiaceae (Nitrobacter, Nitrococcus and Nitrosospira)* organisms contribute significantly to nitrogen geo-recycling, making nitrate readily available for use by plants but also easily leached from the soil or denitrified to nitrogen gas. Other flora characterized here (*Prochlorococcus, Actinmycetes* and *Flavobacterium* spp. including Cytophaga-Bacteroides group) contribute to the marine environment, generating nutrients via photosynthesis or organotrophic mechanisms common in many marine and soil related organisms (Madigan, et al., 2000). It is difficult to compare results generated from this study to other studies, since no studies were found that utilized similar techniques to characterize microbial populations and communities in wetlands. Duncan and Groffman (1994), compared wetland microbial biomass (determined using a chloroform fumigation-incubation method), concluded that biomass was highly variable among samples collected from several diverse sites. Previous research has been conducted characterizing microbial populations and communities in marine, estuarine, rivers, and freshwater lakes (Mullins, et al., 1995; Crump and Baross, 1996; Crump and Berner, 1996; Hiorns, et al., 1997; Methe, et al., 1998; Zwart, et al., 1998a,b; and Crump, et al., 1999), and some common microbes were identified here. However, this conclusion would not benefit from a further comparison of aquatic wetlands to such diverse marine and aquatic systems due to the current lack of comprehensive consistent data to provide a reliable basis for such comparisons. Therefore, this study provides basic information for comparison of results for future studies by our team and/or other researchers. Further research may focus on development and use of DNA spot assays to identify specific bacteria in wetland water samples known to contribute significantly to N, P, and C elemental cycling.

ACKNOWLEDGMENTS

This study was funded by a grant from The National Fish and Wildlife Foundation, administered through The Wetlands Initiative with the support of The Nature Conservancy through their wetland purchase. Their support, guidance, and cooperation are appreciated. PLFA and PCR-DGGE analyses were conducted by Microbial Insights, Rockford, TN (www.microbe.com, E-mail: microbe@microbe.com).

REFERENCES

Crump, C., E. Armbrust, and J. Baross. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. 65(7):3192-3204.

Crump, B.C., and J. Baross. 1996. Particle-attached bacteria and heterotrophic plankton in the Columbia River estuary. Mar. Ecol. Prog. Ser. 138:265-273.

Crump, B.C., and R. Berner. 1996. Marginal marine environments: estuaries, p. 284-311. In Global environment : water, air, and geochemical cycles. Prentice Hall, Englewood Cliffs, NJ.

Duncan, C.P. and P. Groffman. 1994. Comparing microbial parameters in natural and constructed wetlands. Jour. Env. Qual. 23 (Mar.-Apr.):298-305.

Gonzalez, J.M., and M. Moran. 1997. Numerical dominance of a group of marine bacteria in the α-subclass of the class *Proteobacteria* in coastal seawater. Appl. Environ. Microbiol. 63:4237-4242.

Hammer, D.A. 1997. Creating Freshwater Wetlands, 2nd Ed. CRC Lewis Publishers, Boca Raton, FL

Hey, D. and N. Philippi. 1999. A Case for Wetland Restoration. John Wiley and Sons, Pub. , New York, NY.

Hiorns, W.D., B. Methe, S. Nierzwicki-Bauer, and J. Zehr. 1997. Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences. Appl. Environ. Microbiol. 63:2957-2960.

Kadlec, R.H., and R. Knight. 1996. Treatment wetlands. CRC Lewis Publishers, Boca Raton, FL.

Kelley, T.R. and E. Huddleston. 2001. Monitoring of Spunky Bottoms restored wetland in Southern Illinois for biotic and abiotic pollution indicators. Trans., Il. St. Acad. Sci. 94(2):69-78.

Madigan, M., J. Martinko, and J. Parker. 2000. Brock Biology of Microorganisms, Ninth Ed. Prentice Hall, Upper Saddle River, NJ.

Methe, B.A., W. Hiorns, and J. Zehr. 1998. Contrasts between marine and freshwater bacterial community composition: analyses of communities in Lake George and six other Adirondack lakes. Limnol. Oceanogr. 43:386-394.

Mullins, T.D., T. Britschgi, R. Krest, and S. Giovannoni. 1995. Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol. Oceanogr. 43:148-158.

Muyzer, G., E. De Waal, and A. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59(3):695-700.

National Research Council. 1995. Wetlands: Characteristics and Boundaries. National Academy Press, Washington, D.C.

National Water Quality Inventory, 1995. 1996. United States Environmental Protection Agency Office of Water, Washington DC.

Stephen, J.R., Y-J Chang, Y-D, Gan, Peacock, A., Pfiffner, S.M., Barcelona, M.J., White, D.C., MacNaughton, S.J. Microbial Characterisation of JP-4 fuel contaminated site using a combined lipid biomarker PCR_DGGE based approach. Environmental Microbiology, 1999a 1:231-243.

Stephen, J.R., Y-J Chang, Y-D, Gan, Peacock, A., Pfiffner, S.M., Barcelona, M.J., White, D.C., MacNaughton, S.J. Fate of a metal-resistant innoculum in contaminated and pristine soils assessed by denaturing gradient gel electrophoresis. Environmental Toxicology and Chemistry, 1999b. 18(6).

The Condition of Illinois Water Resources 1972 - 1994. 1994. Illinois Environmental Protection Agency, Springfield, IL.

Water Pollution Control Program Plan, Fiscal Year 1996. 1995. Illinois Environmental Protection Agency, Division of Water Pollution Control, Springfield, IL.

Whigham, D.F., D. Dykyjova, and S. Hejny, Eds. 1993. Wetlands of the world I: Inventory, ecology, and management, Vol I, from Handbook of vegetation science Vol. 15/2, H. Lieth, Ed. In Chief. Kluwer Academic Pub., Dordrecht, The Netherlands.

White, D.C., H.C. Pinkart, and D.B. Ringelberg, 1997. Biomass measurements: Biochemical approaches, in Manual of Environmental Microbiology, C.J. Hurst, et al., Editors. ASM Press: Washington. p. 91-101

Zwart, G., W.D. Hiorns, B.A. Methe, M.P. van Agterveld, R. Huismans, S.C. Nold, J.P. Zehr, and H.J. Laanbroek. 1998a. Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. Syst. Appl. Microbiol. 21:546-556.

Zwart, G., R. Huismans, M.P. van Agterveld, Y. Van de Peer, P. De Rijk, H. Eenhoorn, G. Muyzer, E.J. van Hannen, H.J. Gons, and H.J. Laanbroek. 1998b. Divergent members of the bacterial division *Verrucomicrobiales* in a temperate freshwater lake. FEMS Microbiol. Ecol. 25:159-169.

Figure 1: Map of wetlands area and sample locations (well nest and core locations as of 12/31/98).

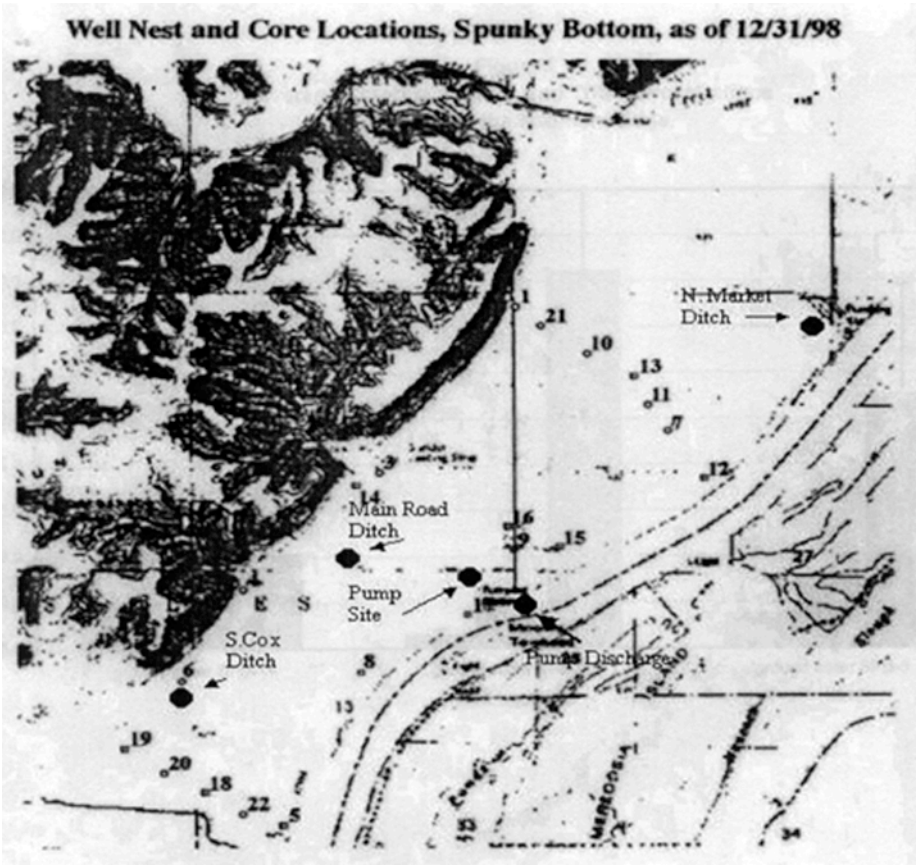
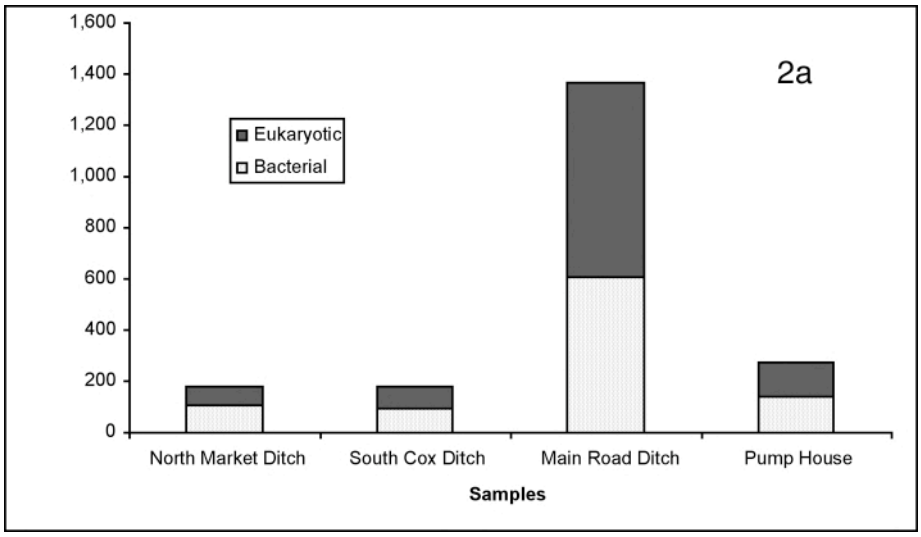


Figure 2a, b. Results of phospholipids fatty acid (PLFA) sample analyses (relative biomass of eukaryotes and bacteria).



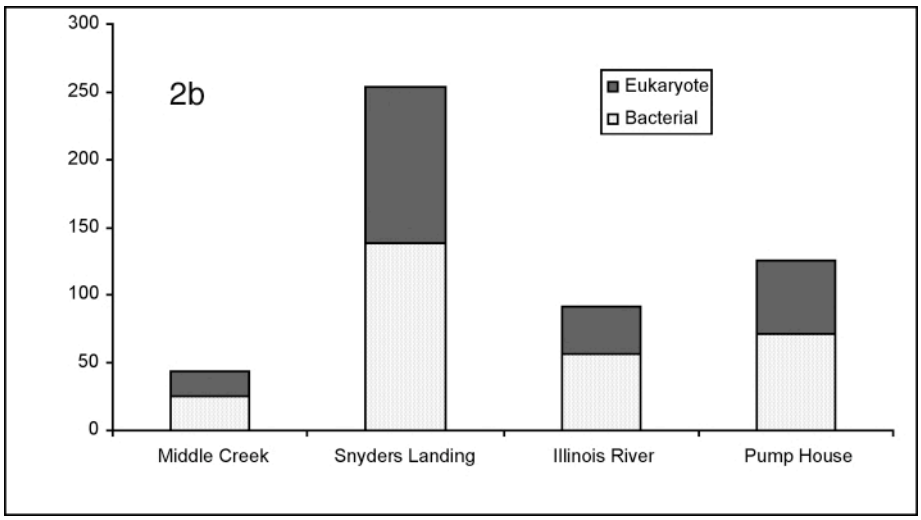
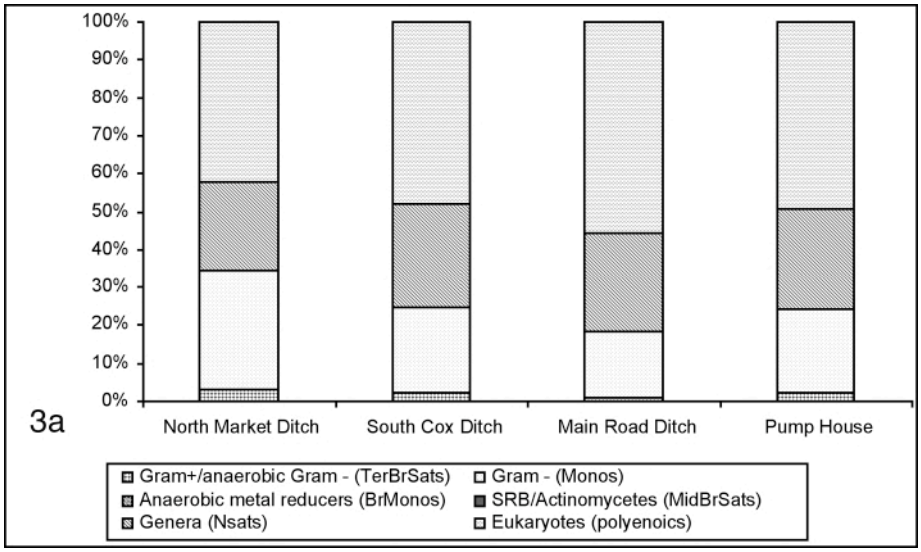


Figure 3a, b. Results of phospholipids fatty acid (PLFA) sample analyses (relative percentages of eukaryotes and bacterial groups).



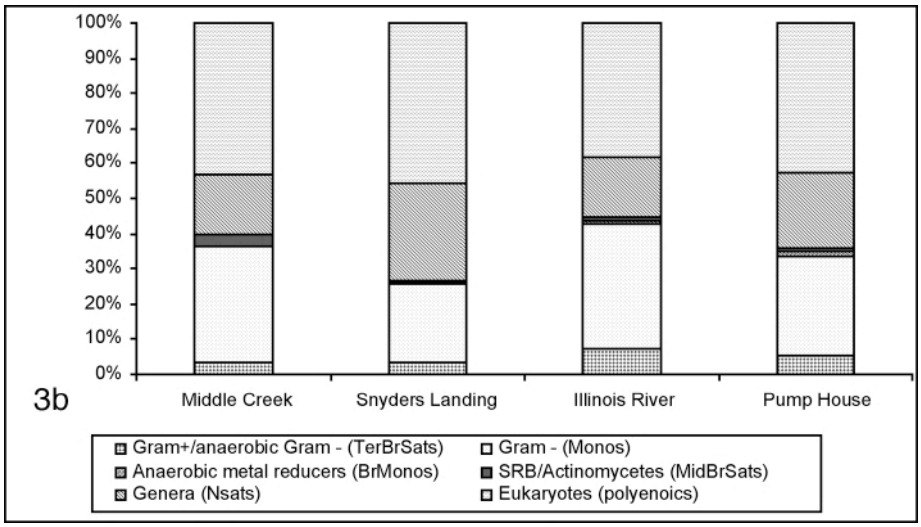


Figure 4a, b. Predominant bands generated from PCR/DGGE analyses selected for identification.