Effects of pH Amendment on Metal Working Fluid Wastewater Biological Treatment Using a Defined Bacterial Consortium

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Abstract: The aim of this study was to determine whether pH amendment of a highly alkaline metal working fluid (MWF) wastewater would improve biological treatment in a bioreactor system following introduction of a bacterial inoculum (comprised of the following strains: *Agrobacterium radiobacter, Comamonas testosteroni, Methylobacterium mesophilicum, Microbacterium esteraromaticum*, and *Microbacterium saperdae*). The pH values tested were 6, 7, 8, and 9. Three replicate batch mode bioreactors inoculated with the bacterial inoculum (plus an abiotic control bioreactor) were operated for each of the four pH conditions. After 14 days, the final mean chemical oxygen demand (COD) reduction at pH 9 was 50 $\pm$ 1.4%; at pH 8, 58 $\pm$ 1.4%; pH 7, 65 $\pm$ 1.0%; and pH 6, 75 $\pm$ 2.7% of the initial COD (approximately 10,000 mg L^{-1}), respectively. Interestingly, within 5 days, the pH in all inoculated bioreactors progressed toward pH 8. However, all abiotic control bioreactors remained at the pH at which they were amended. The fate of the inoculum, determined by denaturing gradient gel electrophoresis (DGGE) and by cluster analysis of the resulting DGGE profiles, revealed that the inocula survived throughout operation of all pH-amended bioreactors. Length-heterogeneity polymerase chain reaction (PCR) was used to track the population dynamics of individual strains. After 7 days of operation, *M. esteraromaticum* was the most abundant population in all bioreactors, regardless of pH. From our findings, it appears necessary to adjust the MWF wastewater from pH 9 to between 6 and 7, to achieve optimal biological treatment rates. © 2004 Wiley Periodicals, Inc.

Keywords: industrial wastewater; metal working fluid; pH; biological treatment; length-heterogeneity PCR; bioaugmentation

INTRODUCTION

Metal working fluids (MWFs) are an essential component of heavy manufacturing facilities (including automotive engine, transmission, and stamping plants). They contribute to the vast majority of organic compounds in wastewater produced by such manufacturing plants (Anderson et al., 2003). Once oil-based MWFs have become operationally exhausted they are typically treated using physical–chemical steps such as ultrafiltration. This established technology is used in many industries, including dyeing, food, and cosmetics. High-pressure cross-flow of the fluid through membrane tubes (pore sizes ranging from 0.01 to 0.1 μm) causes the smaller molecules to permeate through the membranes and larger oil molecules to be retained (Enviro-Wise, 1999). Separation and concentration methods of on-site treatment have been typically applied to MWF effluent. However, increased use of modern water-miscible synthetic formulations has increased the incidence of pollution loads in the final effluent because many of the synthetic components easily permeate through the filtration membrane. These compounds (including antimicrobial agents and other xenobiotics) can be potentially toxic to aquatic life and have caused major problems at sewage treatment works by overloading and killing microorganisms, resulting in substantial fines for the offending company (Bio-Wise, 2000). With the implementation of several proposed UK and European Union directives regulating effluent discharge, the manufacturing industry will have to take increased responsibility for the waste it produces (European Commission, 2000).

A possible solution for dealing with the aqueous effluent produced could be to add a biological treatment step after the initial physical–chemical treatment. However, it is the pore size of membranes used in ultrafiltration ($\leq$0.1 μm) that controls the removal of the microbial biomass, so it is necessary to reinoculate the waste with appropriate microbial communities when it passes to a bioreactor. Bioaugmentation, either with defined microbial cultures of specialized selected strains or inoculation with enriched communities, is still controversial and not a widely accepted technique, and conversely viewed either as a universal solution to bioremediation problems or as useless and expensive (Wagner-Döbler, 2003). However, there

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are many examples of successful bioaugmentation into a variety of habitats and systems, including soil (Beaulieu et al., 2000; Elvang et al., 2001), groundwater (Stucki and Thuer, 1995), and industrial wastewaters (van der Gast et al., 2003b; Wagner-Döbler et al., 2000). Regardless of opinion, physical–chemical treatment of spent MWFs, such as ultrafiltration, removes the microbial biomass along with the waste oil fraction from the wastewater, so it is necessary to inoculate the waste when it passes into the bioreactor system.

In developing a biological treatment strategy, designers must choose between exploiting microorganisms that are indigenous to the polluted habitat or provide some form of bioaugmentation to the system (Graham and Curtis, 2003). Our approach has been to use a combination of these approaches, resulting in construction of a bacterial consortium from the microbial community indigenous to operationally exhausted MWFs for the purpose of degrading aqueous MWF wastewater originating from semi-synthetic fluids. The selection of the consortium member strains was based on three selection criteria: (i) degradative ability; (ii) tolerance to cocontaminants; and (iii) spatial and temporal abundance in operationally exhausted MWFs (van der Gast et al., 2002, 2003a).

Inocula survival is an essential goal if they are to be effective in biotechnological applications in which the microorganisms are exposed to a variety of stresses such as toxic metals, xenobiotics, and the fluctuating nature of the habitat (temperature and pH) (Timmis and Pieper, 1999). In the context of this study, in-use MWFs had an alkaline pH, typically between 9 and 11. This high alkalinity is important in maximizing the performance of a fluid, because lower pH values can lead to corrosion and biodeterioration of in-use fluids (Johnson and Nash, 1986). Even after a MWF has become operationally exhausted, the resulting wastewater maintains a high pH (>9). It has been reported that microorganisms in industrial wastewater bioreactors can function over a wide range of pHs (6 to 9) (Bramucci and Nagarajan, 2000; McKinney, 2000). Toward this end, the main aims of the current study were: (1) to determine if pH manipulation of the highly alkaline (pH > 9) MWF wastewater would improve biodegradation by an introduced bacterial inoculum; and (2) to assess the fate and performance of the bacterial consortium under different pH conditions in bioreactor systems.

MATERIALS AND METHODS

Metal Working Fluid (MWF) Wastewater

MWF aqueous effluent, provided by the Production Engineering Research Association (Melton Mowbray, UK), was primarily from a semisynthetic fluid (Hysol X, Castrol, Ltd., UK) used as a coolant and lubricant in large-scale, continuous metal working processes. Before biological treatment, the MWF was treated by ultrafiltration (UF), leaving a permeate containing the following main chemical constituents: benzotriazole, boron, citric acid, formaldehyde, monoethanolamine, morpholine, and triethanolamine. For bioreactor studies, the Hysol X permeate was filtered twice through 0.2-μm pore-size filters (35-mm diameter, Millipore, UK) to remove any potential background microbial populations that may have colonized the fluid between the UF and biological treatment steps.

Amendment and Measurement of pH

Four pH conditions (pH 6, 7, 8, and 9) were investigated in this study. When unamended, the pH of the MWF was 9. The pH was amended by adding hydrochloric acid for each condition prior to the start of bioreactor operation. Bioreactor sample pH values were measured using a pH electrode (Gelplas, BDH, Quebec, Canada) connected to a pH monitor (Model 3305, Jenway, Ltd., Essex, UK) at time of sampling.

Bioreactor Operation

MWF wastewater studies were performed in sealed bubble-column bioreactors (5-L total volume). For each pH condition, three replicate bioreactors were operated, with a fourth reactor used as an abiotic control. All bioreactors were run under batch suspension conditions (14-day duration), using working volumes of 4.5 L. Air flow within the bioreactors was maintained at 3.3 L min^{-1}, using aquarium air pumps and air spargers (Fisher Scientific, UK). Reactor temperature was maintained at 28 ± 1°C, using water-heated jackets. Samples of 10 mL were taken for analyses at daily intervals over a 14-day period.

Inoculation Conditions and Bacterial Consortia Construction

The bacterial consortium employed in this study was constructed by screening large numbers of waste Hysol X MWF samples, and selecting the most spatially and temporally distributed strains with the best degradative performance, following a selection strategy described previously (van der Gast et al., 2002). The consortium consisted of five bacterial strains: *Agrobacterium radiobacter* (designated strain 5-BA-A), *Comamonas testosteroni* (1-BTZ-O), *Methylobacterium mesophilicum* (20-BTZ-N), *Microbacterium esteraromaticum* (15-BTZ-N), and *Microbacterium saperdae* (1-TEA-C). These five strains met all three selection criteria: (1) they were ubiquitous in spatially and temporally separate samples; (2) they could degrade the chemical constituents of the MWF; and (3) they were tolerant to cocontaminants. The five strains were inoculated separately into 250-mL conical flasks containing 100 mL of tryptic soy broth (10% [v/v] Difco, UK) and prefiltered (using a 0.2-μm pore-size filter, Millipore, UK)

MWF wastewater (3% [v/v]). The individual cultures were incubated at 28°C in an orbital shaker for 12 h (cell counts approximated to 10^7 cells mL^{-1}). The cell suspensions were removed and resuspended in MWF wastewater, mixed together, and added as a 10% (v/v) inoculum into the bioreactors.

Chemical Oxygen Demand

Pollution load analyses, expressed by chemical oxygen demand (COD), were measured using a LASA 100 mobile laboratory photometer (Dr. Lange, UK) with COD cuvette test kits (range 5000 to 60,000 mg L^{-1}, Dr. Lange, UK). MWF samples were prefiltered using a 0.2-μm pore-size membrane (Millipore, UK) and analyses were performed according to the manufacturer's instructions.

Total Count Microscopy

Cells were fixed with a fresh 1% (w/v) final concentration of paraformaldehyde (Sigma, Poole, UK) in phosphate-buffered saline solution. DAPI (4′, 6′-diamidino-2-phenylindole, Sigma) at 2-μg mL^{-1} final concentration was added to fixed samples and left for 10 min at room temperature. Samples were filtered using 0.2-μm pore-size filters (25-mm diameter, Poretics Corp.) by applying a slight vacuum. After filtration, filters were mounted in oil and observed immediately by epifluorescence microscopy (Eclipse E600, Nikon, Japan).

Statistical Treatment

To examine the relationships between bacterial abundance and the COD of MWF wastewater in bioreactors, all data were analyzed for significant correlations using MINITAB software (MINITAB 12, Minitab, Inc.). Only those relationships that were significant at the 5% level ($P < 0.05$) and consistently detected over a minimum of five data points were described.

DNA Extraction, 16S rDNA Amplification, and DGGE Analyses

The genetic diversity of the whole bacterial community contained in bioreactor samples was determined. Total DNA samples were extracted, PCR products amplified, and the fragments analyzed by denaturing gradient gel electrophoresis (DGGE), as described previously (van der Gast et al., 2001). DGGE profile analyses were performed using Phoretix 1D analysis software according to the manufacturer's instructions (Phoretix Intenational, UK). Dendrograms for comparisons of DGGE samples were generated by an unweighted pair group method, using mathematical averages algorithm programs integral to the commercial software.

Length-Heterogeneity PCR (LH-PCR) Amplification of MWF Sample DNA Extracts

DNA extractions, for amplification of the 16S rDNA gene from bioreactor samples, were performed as described previously (van der Gast et al., 2001). The 16S rDNA gene was amplified from extracted DNA, originating from direct MWF extracts using a length-heterogeneity approach (Whiteley et al., 2003) that accounted for the variable 16S rDNA lengths, which were due to prokaryote-group-specific insertion/deletion events between primer 43f and 519r. The amplification consisted of the *Bacteria* primer set, "43f" [TCA GA (A/T) (C/T) G A ACG CTG GCG G] and "519r" [GTA TTA CCG CGG CTG GCT G], labeled with Beckman Dye4 (Proligo, France) to produce an approximate 500-basepair (bp) fluorescently labeled PCR fragment. These were used in the following 50-μL volume reaction: 43f and 519r primer, 10 p*M*; dNTPs, 50 μ*M* of

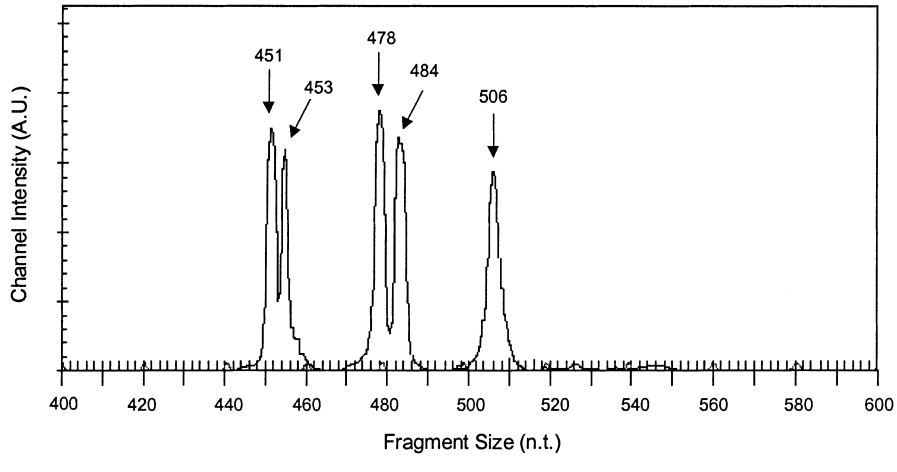

Figure 1. Representative LH-PCR trace of members of the bacterial consortium studied. Fragments at 451 bp correspond to *Agrobacterium radiobacter*; 453 bp, *Methylobacterium mesophilicum*; 478 bp, *Microbacterium esteraromaticum*; 484 bp, *Microbacterium saperdae*; and 506 bp, *Comamonas testosteroni*, respectively.

each; 1 unit of Taq-polymerase (Sigma), 1.5 m*M* $MgCl_2$, and 5 to 20 ng of template, respectively. The thermal cycling employed consisted of one cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, followed by 72°C for 2 min. Amplification products were examined with a 2% (w/v) agarose gel to confirm a 500-bp amplification product before LH-PCR.

LH-PCR Fragment Analysis and Calibration

One microliter of amplified 16S rDNA, taken directly from the PCR reaction, was analyzed using a sequencer (Model CEQ2000, Beckman-Coulter Corp.) to ascertain the distribution of fragment sizes within the PCR reaction. The distribution of fragment sizes from different bacterial groups was found to be within the range 450 to 530 bp for the primer set involved. The fragment distributions were deconvolved by peak detection and size analysis using standard parameters available with CEQ8000 sequencing software, and using an internal 600-bp ladder for accurate sizing (Beckman-Coulter). Specifically, analysis of individual consortium member strains was performed to ascertain their characteristic fragment lengths between the 43f and 519r primer sites (Fig. 1).

RESULTS

Changes in MWF Wastewater pH

Three replicate bioreactors inoculated with the bacterial consortium (plus an abiotic control bioreactor) were operated for each of the four pH conditions (6, 7, 8, and 9). Within 5 days, the pH in all inoculated bioreactors progressed toward pH 8 (Fig. 2). However, all abiotic control bioreactors remained at the pH at which they were amended.

Chemical Oxygen Demand

Reduction in pollution load was measured by COD with an initial effluent COD value (day 0) of approximately

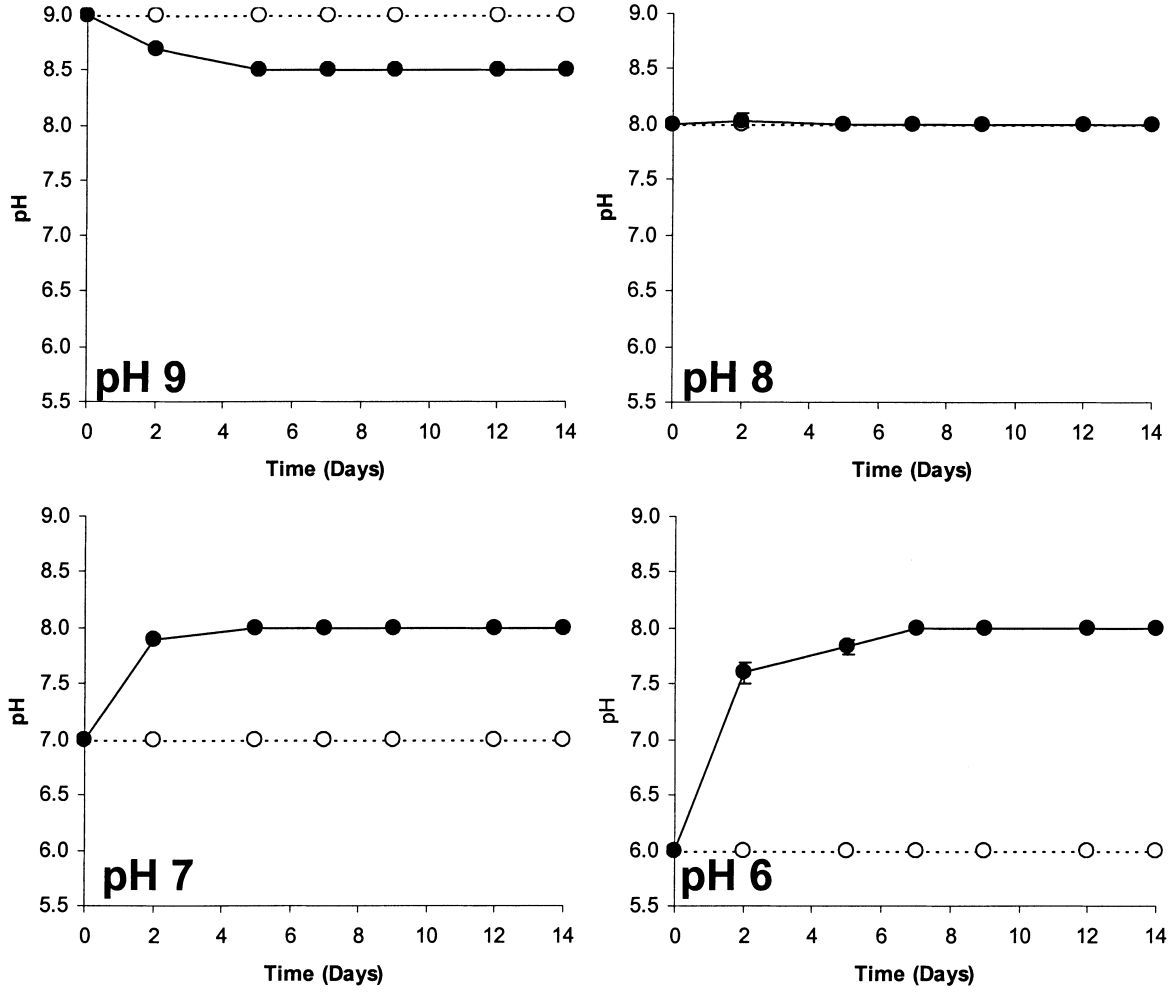

Figure 2. Fluctuation in pH in pH-amended bioreactors over 14 days of batch reactor operation. Bioreactors inoculated with bacterial consortia (---○---) and abiotic control bioreactors (—●—) are shown. Error bars represent standard deviation of the mean (n = 3).

10,000 mg L^{-1} (Fig. 3). COD values in abiotic reactors varied little from the initial 10,000-mg L^{-1} COD load throughout the study. The final mean COD reduction for inoculated bioreactors (n = 3) at pH 9 was 50 ± 1.4%; at pH 8, 58 ± 1.4%; pH 7, 65 ± 1.0%; and pH 6, 75 ± 2.7% of the initial values, respectively.

Genotypic Analysis Determined by 16S rDNA Denaturing Gradient Gel Electrophoresis

DGGE analyses of the amplified 16S rDNA from pH-amended bioreactors, sampled at days 0, 7, and 14 of operation, revealed that all profiles were highly conserved with >90% similarity (Fig. 4). In fact, with the exception of a single sample of pH 9 (replicate reactor A) taken at day 14, all other samples clustered at >94% similarity. In addition, no DNA was extracted or amplified from any of the abiotic pH-amended bioreactors.

Consortium Abundance and Structure Determined by DAPI Counts and LH-PCR Analyses of Total Community DNA

Measures of bacterial abundance were determined by total DAPI counts using microscopy. Total DAPI counts in pH 6 and 7 bioreactors increased from approximately 3×10^7 to 4×10^9 cells mL^{-1} over the 14-day operation (Fig. 5). Bacterial abundance within pH 8 and 9 bioreactors varied little, ranging from approximately 3×10^7 to 8×10^7 cells mL^{-1}. No cells were detected in any of the abiotic control bioreactors. When pollution load (COD) was related to total DAPI count, total cell counts were found to correlate significantly ($P < 0.05$) only in pH 6- and pH 7-amended bioreactors (Fig. 6). Bacterial counts in these bioreactors exhibited inverse linear relationships.

The population dynamics of the introduced microbial consortium within the bioreactors were monitored using LH-PCR. To differentiate between different peaks within

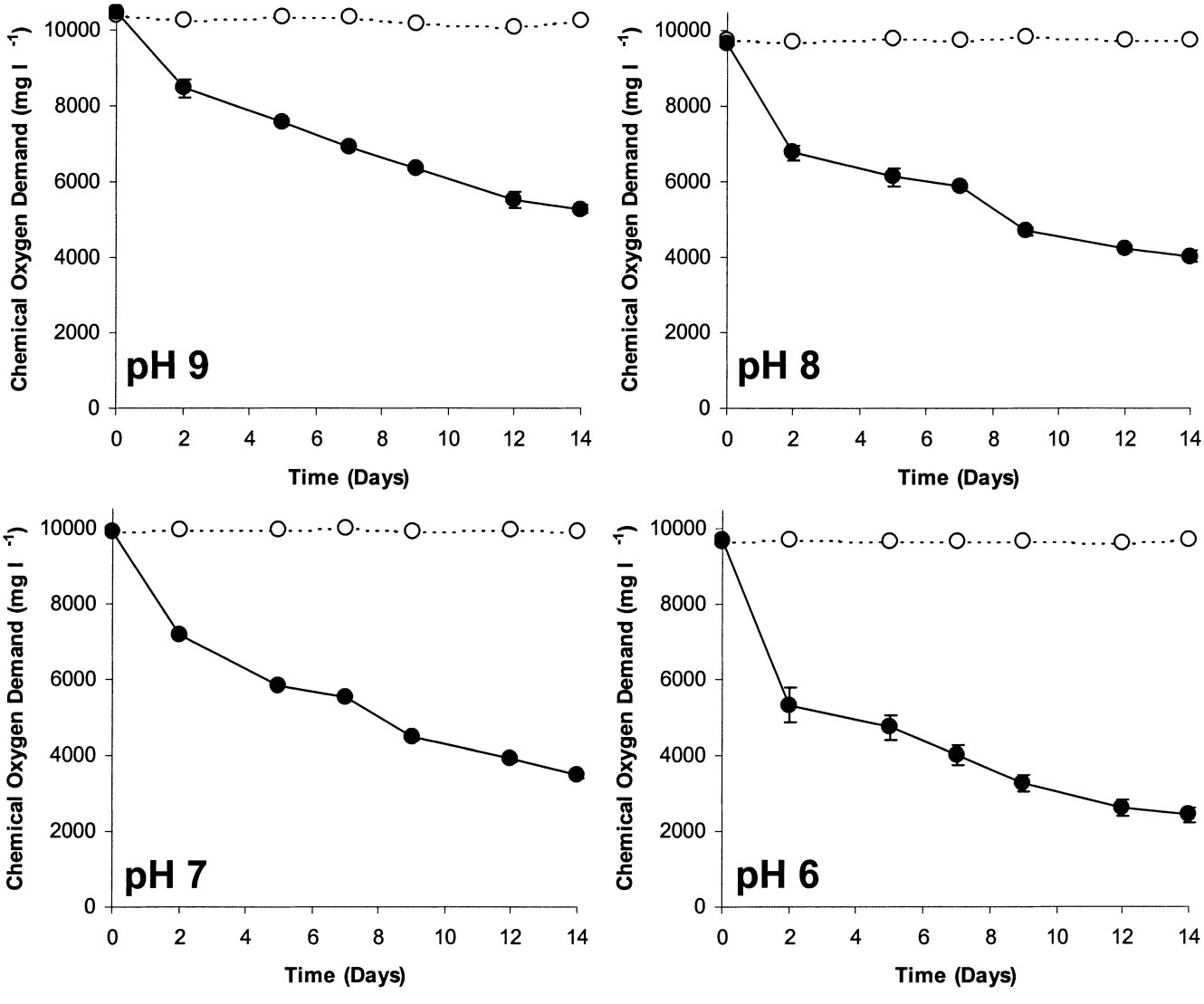

Figure 3. Reduction of pollution load (chemical oxygen demand) in pH-amended bioreactors over 14-day batch operation. Inoculated bioreactors (---○---) and abiotic control bioreactors (—●—) are shown. Error bars represent standard deviation of the mean (n = 3).

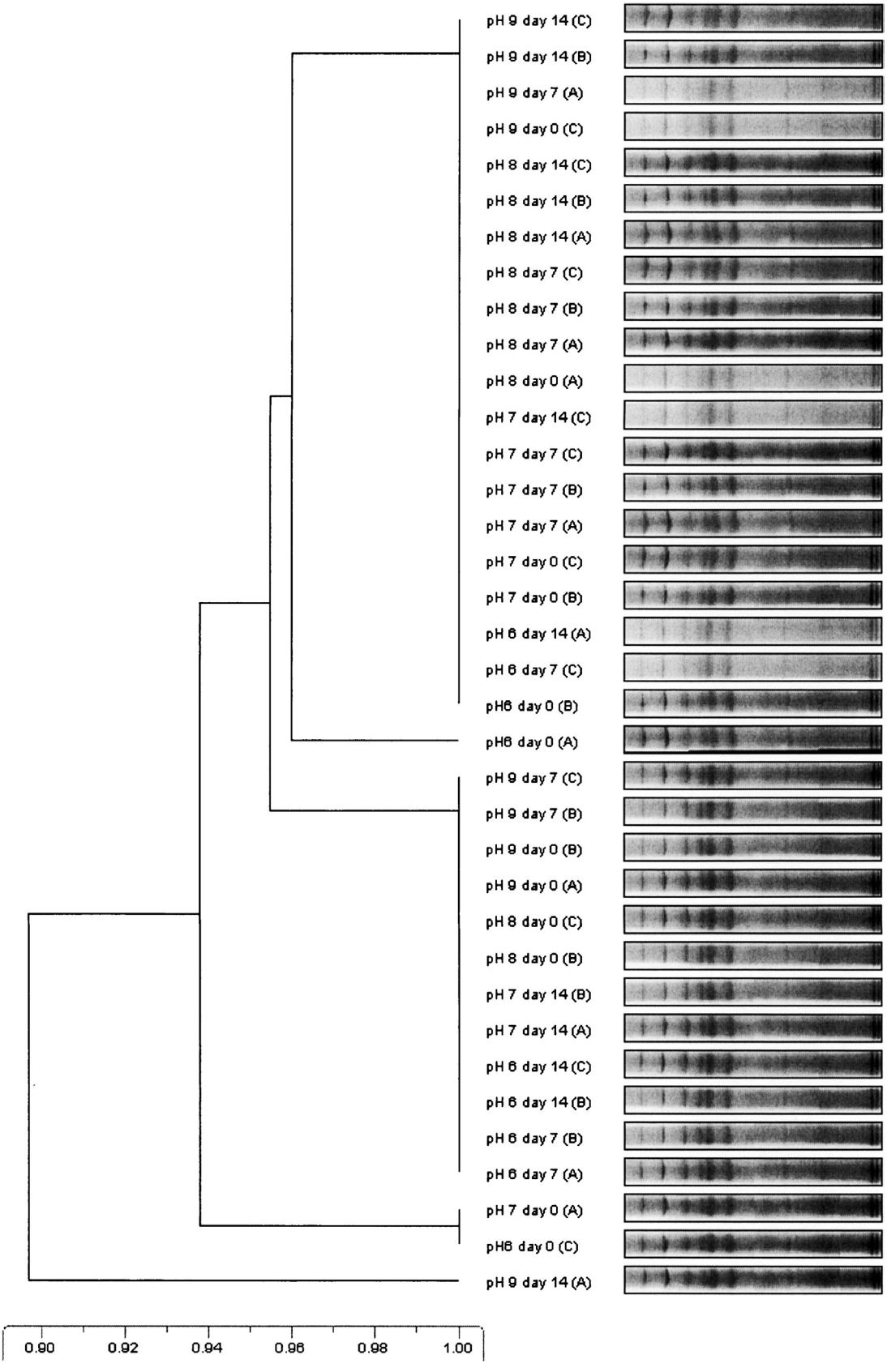

Figure 4. Dendrogram of DGGE profiles in bioreactor samples (at days 0, 7, and 14) at pH 6, 7, 8, and 9, using unweighted pair group mathematical averages showing the relationship between profiles by pairwise comparison of band presence and position. (A), (B), and (C) are replicate bioreactors for each pH condition.

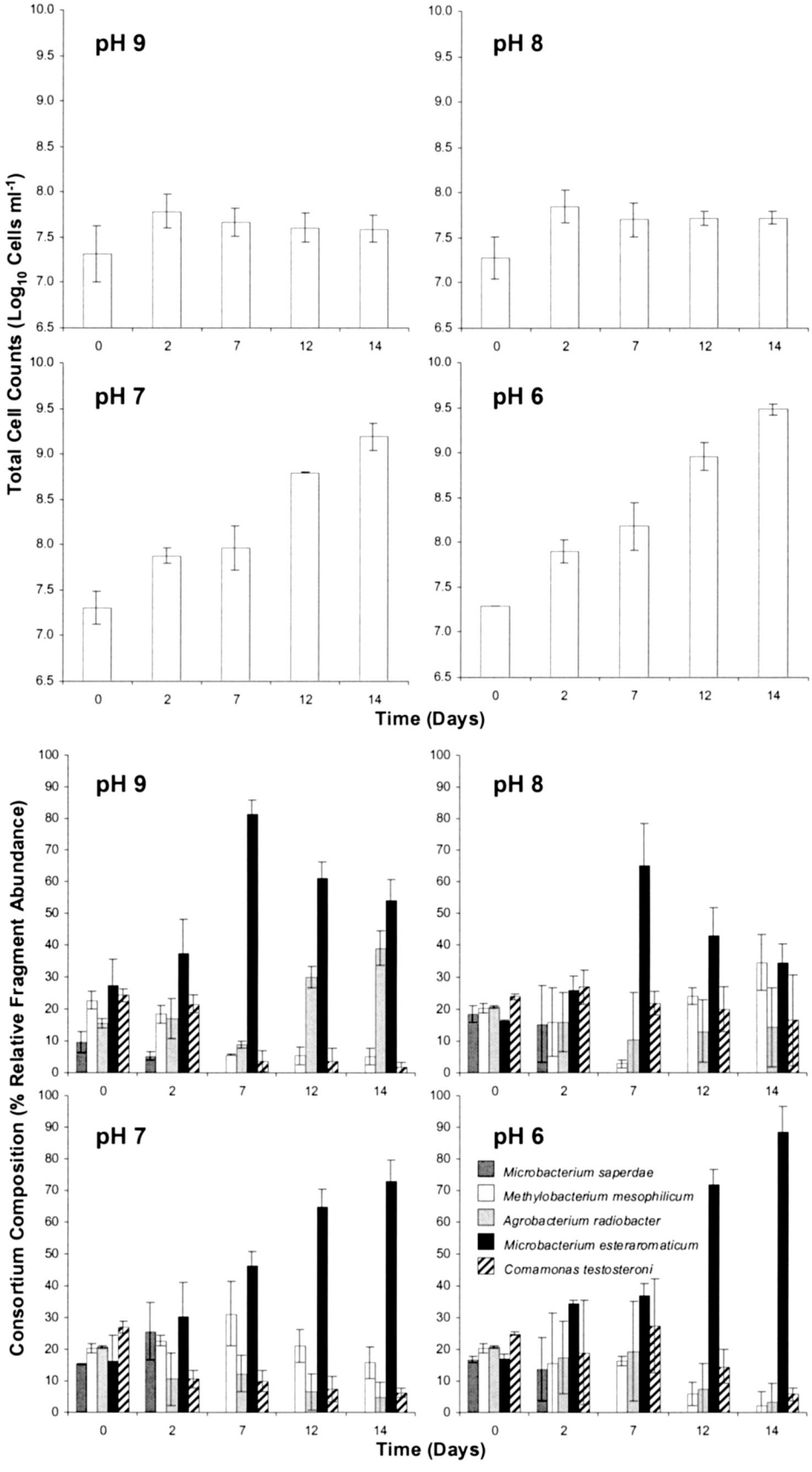

Figure 5. Total cell counts obtained by DAPI staining and bacterial consortium composition determined by length-heterogeneity PCR (expressed as percent relative LH-PCR fragment abundance), within pH-amended bioreactors over 14-day operation. Error bars represent standard deviation of the mean ($n = 3$).

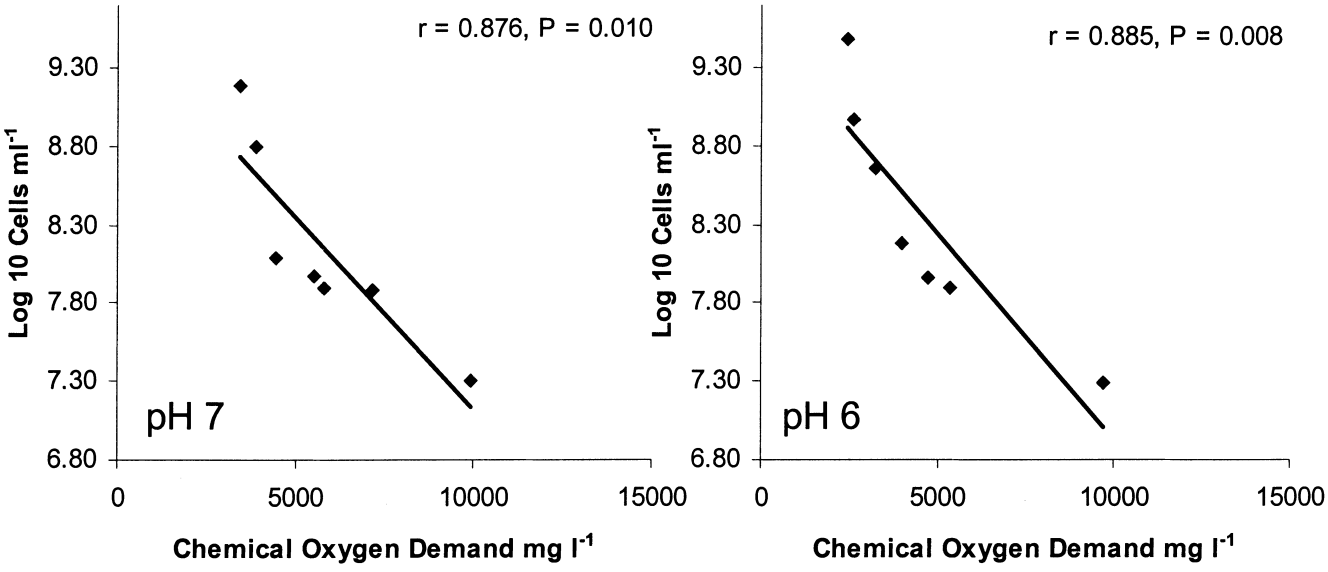

Figure 6. Relationships between total cell counts and chemical oxygen demand (COD) in pH 7 and pH 6 bioreactors. The relationships presented are significant at $P < 0.05$.

LH-PCR traces of samples containing the whole bacterial consortium, analyses were performed on individual consortium strains to ascertain their corresponding fragment sizes (Fig. 1). To study temporal consortium strain dynamics in a quantitative manner, LH-PCR peak heights were expressed as the percentage relative peak abundance in a LH-PCR sample trace (Fig. 5). LH-PCR analyses revealed that all strains were detectable in all bioreactors with the exception of the *Microbacterium saperdae* strain, which was not detected after 7-day bioreactor operation. Only peaks corresponding to the consortium strains were detected throughout the study and no other peaks were detected from any of the abiotic pH-amended bioreactors, indicating that only consortium populations were present in the bioreactors. From day 7, in all inoculated bioreactors, the most abundant LH-PCR peaks corresponded to the *Microbacterium esteraromaticum* population (Fig. 6). The percentage relative fragment abundance of this strain peaked on day 7 in pH 9 and pH 8 bioreactors, reaching a level of 81.7% (±4.3%) and 65.0% (±8.5%), respectively. In contrast, in the pH 7- and pH 6-amended bioreactors, the *M. esteraromaticum* corresponding peaks reached a maximum of 73.0% (±5.6%) and 88.4% (±8.5%) of the population detected after 14-day operation, respectively.

DISCUSSION

Bioaugmentation remains a controversial approach and is still not universally accepted for treating industrial wastewaters. However, in attempts to biologically treat metal working fluid wastewaters it is necessary to inoculate the effluent, as it is typically ultrafiltered (≤0.1-μm membrane pore size), and thus remove the microbial biomass in addition to the high-molecular-weight oil molecules (Enviro-Wise, 1999). Biological systems designed for metal working fluid wastewater treatment have typically been inoculated with undefined microbial communities from activated sludge (Baker et al., 1983; Kim et al., 1992a, 1992b; Roberts et al., 2000). Conversely, bioaugmentation with carefully selected consortia may improve the possibility of re-creating more reproducible systems that both enhance degradative ability and, by preemptive colonization, can prevent invasion by opportunistic strains that possess little degradative ability or are pathogenic (van der Gast et al., 2002).

In previous studies, we demonstrated that selection of strains, based on understanding of the diversity of indigenous MWF populations, can lead to more effective processing of MWF wastewaters by bioaugmentation than by using uncharacterized communities from activated sludge (van der Gast et al., 2003b, 2004). The main aim of this study was to determine whether pH amendment of an MWF wastewater would enhance its degradation by a carefully constructed bacterial consortium that contained strains demonstrated to be abundant and catabolically effective. The pH range was selected based on reports that microorganisms in industrial wastewaters can function typically between pH 6 and 9 (Bramucci and Nagarajan, 2000; McKinney, 2000). In addition, previous research has demonstrated that fungal and yeast growth is promoted below this level, which could lead to proliferation of undesired microorganisms with little degradative ability in a bioreactor system (Hill, 1978; Rossmoore and Holtzman, 1974; von Holy, 1989). Reduction of pollution load (COD) was greater in bioreactors with lower pH, with 75% COD removal after 14-day operation in pH 6-amended bioreactors (Fig. 3). We are confident that the decline of COD and the regulation of pH in all inoculated bioreactors toward pH 8 was due to the consortia alone as no change in COD or

pH was detected in any of the abiotic bioreactors (Fig. 2). A possible explanation as to why the strains regulated the pH toward 8 may be a survival mechanism/stress response to the harsh nature of the MWF wastewater. It has been reported previously that microorganisms in soil or water can dramatically alter the pH of their own environment by metabolic production (Slonczewski, 2000). However, to our knowledge, this has never been previously observed or reported in bioreactor systems for treating industrial waste. Another explanation could be that breakdown products of nitrogen-containing compounds within the MWF wastewater, such as monoethanolamine and triethanolamine, shift the pH toward 8 due to the release of ammonia. Both possibilities will be subject to further investigation.

The fate of the bacterial consortium determined by denaturing gradient gel electrophoresis (DGGE) and by cluster analyses of the resulting DGGE profiles revealed that the inocula survived throughout operation of all pH-amended bioreactors (Fig. 4). All community profiles were strikingly similar (>90% similarity), revealing the highly conserved nature of the consortium. Although it is an effective technique for detecting total diversity, DGGE does not allow determination of individual strain quantities. Toward this end, we used LH-PCR to track the population dynamics of the individual strains throughout operation of the pH-amended bioreactors. After 7-day operation, *Microbacterium esteraromaticum* was the most abundant strain in all bioreactors, regardless of pH. Conversely, *Microbacterium saperdae* was not detected after this time in any of the bioreactors. One possible explanation for this could that be the *M. esteromaticum* strain out-competed and was fitter than the *M. saperdae* strain, as both strains would have similar nutrient and growth requirements. All consortium members were present up to day 7 of the bioreactor run and were in approximately equal abundance up to day 2, which coincided with the greatest drop in COD in the bioreactors (Fig. 5). Therefore, it is possible that the *M. esteraromaticum* strain was more adept at utilizing the chemical components of the MWF wastewater at lower concentrations after the majority of the MWF wastewater COD had been removed.

To our knowledge, this is the first study aimed at determining the effects of pH amendment of MWF wastewater on the biodegradative performance of a bacterial inoculum. Most heterotrophic bacteria favor a pH near neutrality (Leahy and Colwell, 1990). This is in agreement with our findings and those of previous research into optimal pH values. An optimal pH of approximately 6.7 was observed in one study of biodegradation of the organophosphate insecticide chlorpyrifos in soils with a pH ranging from 4.7 to 8.4 (Singh et al., 2003). Likewise, the optimal pH of 7.8 was observed in a study into the mineralization of oily sludge, in soil ranging from pH 5.0 to 7.8 (Dibble and Bartha, 1979). From our findings, it would appear necessary to adjust MWF wastewater pH from alkaline (>9) to between 6 and 7 for increased biological treatment rates. In this study, we only amended MWF pH prior to bioreactor operation. We now plan to determine the effects of pH maintained at 6 in long-term bioreactor studies under sequence batch operation.

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References

Anderson JE, Kim BR, Mueller SA, Lofton TV. 2003. Composition and analysis of mineral oils and other compounds in metalworking and hydraulic fluids. Crit Rev Environ Sci Technol 33:73–109.

Baker CA, Claus GW, Taylor PA. 1983. Predominant bacteria in an activated sludge reactor for the degradation of cutting fluids. Appl Environ Microbiol 46:1214–1223.

Beaulieu M, Becaert V, Deschenes L, Villemur R. 2000. Evolution of bacterial diversity during enrichment of PCP-degrading activated soils. Microbial Ecol 40:345–355.

Bio-Wise. 2000. A guide to biological treatment for metalworking fluids disposal. Report No. DTI/BW/25/2000/NP, Department of Trade and Industry.

Bramucci MG, Nagarajan V. 2000. Industrial wastewater bioreactors: sources of novel microorganisms for biotechnology. Trends Biotechnol 18:501–505.

Dibble JT, Bartha R. 1979. Effect of environmental parameters on the biodegradation of oil sludge. Appl Environ Microbiol 37:729–739.

Elvang AM, Westerberg K, Jernberg C, Jansson JK. 2001. Use of green fluorescent protein and luciferase biomarkers to monitor survival and activity of *Arthrobacter chlorophenolicus* A6 cells during degradation of 4-chlorophenol in soil. Environ Microbiol 3:32–42.

Enviro-Wise. 1999. Optimising the use of metalworking fluids. Report No. GG 199. Department of Trade and Industry.

European Commission. 2000. Council Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Communities No. L327.

Graham DW, Curtis TP. 2003. Ecological theory and bioremediation. In: Head IM, Singleton I, Milner MG, editors. Bioremediation: a critical review. Wymondham, Norfolk, UK: Horizon Scientific Press. p 61–92.

Hill EC. 1978. Biodegradation of hydrocarbon based products in industrial use. In: Watkinson RJ, editor. Developments in biodegradation of hydrocarbons. Barking, UK: Applied Science. p 201–225.

Johnson TL, Nash JC. 1986. Primary amine alternatives to triethanolamine in metalworking fluid formulations. Lubr Eng 42:402–407.

Kim BR, Anderson SG, Zemla JF. 1992a. Aerobic treatment of metal-cutting-fluid waste-water. Water Environ Res 64:258–262.

Kim BR, Zemla JF, Anderson SG, Stroup DP, Rai DN. 1992b. Anaerobic removal of COD in metal-cutting-fluid waste-water. Water Environ Res 64:216–222.

McKinney RE. 2000. Wastewater treatment, municipal. In: Lederberg J, editors. Encyclopedia of microbiology. San Diego, CA: Academic Press. p 870–883.

Leahy JG, Colwell RR. 1990. Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315.

Roberts JA, Sutton PM, Mishra PN. 2000. Application of the membrane biological reactor system for combined sanitary and industrial wastewater treatment. Int Biodeter Biodegr 46:37–42.

Rossmoore HW, Holtzman GH. 1974. Growth of fungi in cutting fluids. Dev Indust Microbiol 15:273–280.

Singh BK, Walker A, Morgan JAW, Wright DJ. 2003. Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. Appl Environ Microbiol 69:5198–5206.

Slonczewski JL. 2000. pH stress. In: Lederberg J, editor. Encyclopedia of microbiology. San Diego, CA: Academic Press. p 625–632.

Stucki G, Thuer M. 1995. Experiences of a large-scale application of 1,2-dichloroethane degrading microorganisms for groundwater treatment. Environ Sci Technol 29:2339–2345.

Timmis KN, Pieper DH. 1999. Bacteria designed for bioremediation. Trends Biotechnol 17:201–204.

van der Gast CJ, Whiteley AS, Thompson IP. 2004. Temporal dynamics and degradation activity of an bacterial inoculum for treating waste metal-working fluid. Environ Microbiol 6:254–263.

van der Gast CJ, Knowles CJ, Wright MA, Thompson IP. 2001. Identification and characterisation of bacterial populations of an in-use metal-working fluid by phenotypic and genotypic methodology. Int Biodeter Biodegr 47:113–123.

van der Gast CJ, Knowles CJ, Starkey M, Thompson IP. 2002. Selection of microbial consortia for treating metal working fluids. J Indust Microbiol Biotechnol 29:20–27.

van der Gast CJ, Whiteley AS, Lilley AK, Knowles CJ, Thompson IP. 2003a. Bacterial community structure and function in a metal-working fluid. Environ Microbiol 5:453–461.

van der Gast CJ, Whiteley AS, Starkey M, Knowles CJ, Thompson IP. 2003b. Bioaugmentation strategies for remediating mixed chemical effluents. Biotechnol Progr 19:1156–1161.

von Holy A. 1989. Susceptibility of metalworking fluids to microbiological contamination in service. S Afr J Sci 85:149–151.

Wagner-Döbler I. 2003. Microbial inoculants: snake oil or panacea? In: Head IM, Singleton I, Milner MG, editors. Bioremediation: a critical review. Wymondham, Norfolk, UK: Horizon Scientific Press. p 259–289.

Wagner-Döbler I, von Canstein HF, Li Y, Timmis KN, Deckwer WD. 2000. Removal of mercury from chemical wastewater by microorganisms in technical scale. Environ Sci Technol 21:4628–4634.

Whiteley AS, Griffiths RI, Bailey MJ. 2003. Analysis of the microbial functional diversity within water stressed soil communities by flow cytometric. J Microbiol Meth 54:257–267.