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Temporal dynamics and degradation activity of an bacterial inoculum for treating waste metal-working fluid

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Summary

In order for established bioreactors to be effective for treating chemically mixed wastes such as metal working fluids (MWF) it is essential that they harbour microbial populations that can maintain sufficient active biomass and degrade each of the chemical constituents present. In this study we investigated the effectiveness of a bacterial consortium composed of four species (*Clavibacter michiganensis*, *Methylobacterium mesophilicum*, *Rhodococcus erythropolis* and *Pseudomonas putida*), assembled on the basis of their apparent ubiquity in waste MWF, degradation ability and tolerance to fluctuating chemistry of the waste. The temporal dynamics of the inoculum and its effects on the fate of individual chemical components of the waste were studied, by regular sampling, over 400 h. Using a complementary approach of culture with chemotaxonomic (FAME) analysis and applying group specific probes (FISH), the inoculum was found to represent a significant component of the community in bioreactors with and without presence of indigenous MWF populations. In addition, the reduction in the COD by the consortium was approximately 85% of the total pollution load, and 30–40% more effectively than any other treatment (indigenous MWF community alone or activated sludge). Furthermore, all the chemical constituents, including the biocide (a formaldehyde release agent) demonstrated >60% reduction. Many chemical components of the MWF proved to be recalcitrant in the other treatments. The results of this study confirm that assemblage of an inoculum, based on a comprehensive knowledge of the indigenous microbial community, in the target habitat, is a highly effective way of selecting microbial populations for bioaugmentation of bioreactors.

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Introduction

Manufacturing practices generate huge quantities of chemically heterogeneous wastes that must be treated before leaving the site. Many of the current disposal methods (evaporation, landfill or incineration) are increasingly non-viable options because they are either uneconomical, inefficient or environmentally unacceptable. Because of these limitations, there is increasing interest in biological treatment systems for processing industrial wastes, such as metal working fluids (MWF). Metal working fluids are used as coolants and lubricants in heavy industry, for metal cutting and grinding, and drilling operations. They are chemically complex and specifically formulated to include constituents that improve lubrication, and cooling performance, and inhibit metal corrosion and microbial biodeterioration (biocides). Such mixtures render MWF potentially toxic to the environment. The annual growth rate in sales of MWF across Europe is estimated to be 9–12%, with an estimated world-wide generation of waste of 22.4×10^9 litres annually (Bio-Wise, 2000). The United Kingdom produces 400 million litres year^{-1} of waste MWF alone, with estimated yearly disposal costs of between £8 and £16 million (Bio-Wise, 2000). At present the majority of global waste MWF is incinerated, sent to landfill sites or treated at sewage works. However, with the imminent implementation of several proposed US Federal and European Union directives regulating effluent, incinerator and landfill discharges the present cost effective options for the waste management of spent MWF will no longer be viable (Anonymous, 1995; 2001; European Union 2000a,b).

One solution to this disposal problem is on-site biological treatment of waste MWF, using bioreactor systems. Biological treatment of waste MWF by microorganisms in bioreactor systems has been investigated by several researchers (Baker *et al.*, 1983; Kim *et al.*, 1994; Roberts *et al.*, 2000; Taylor, 2001). Currently bioreactors established for disposing of MWF are commonly operated using

a 'black box' approach, typically inoculated with undefined microbial communities from sewage, a notoriously heterogeneous and potentially dangerous source, because it is likely to harbour potential pathogens (Hamer, 1997). As an alternative, bioaugmentation with carefully selected strains may improve the opportunity to create more reproducible systems that enhance degradative ability, and by pre-emptive colonization, reduce invasion by opportunistic populations with little degradative ability. Evidence suggests mixed inoculation would be better suited for MWF treatment, as bioaugmentation of multisubstrate habitats (such as wastewater, groundwater, soil or slurry) with pure cultures, has been typically proven ineffective (Goldstein *et al*., 1985; Bouchez *et al*., 2000). Introduction of acclimatised microbial populations, isolated from a contaminated habitat or target waste, may be a better option (Beaulieu *et al*., 2000; Vogel and Walter, 2001), as these populations have been shown to be more resistant to extreme ambient conditions and even predation (Fewson, 1988; Otte *et al*., 1994; Hamer, 1997).

In previous studies we examined the performance and fate, in one sampling occasion, of four bacterial strains introduced into bioreactors to bioaugment the treatment of operationally exhausted MWF. At the end of the study (100 h) the consortium was found to have reduced the pollution load (COD) by 80%, and the introduced strains found to persist (van der Gast *et al*., 2003). Although encouraging, in order to confidently determine the potential of inoculated bioreactors in scaled-up industrial applications, it was desirable to undertake in depth temporal studies of the inocula over an extended period. If bioreactors for treating of chemically mixed wastes, such as MWF, are to become practical options, it is essential that inoculated strains can be demonstrated to be more effective than systems containing only indigenous microbial populations. In addition, they must be able to persist in the fluctuating chemical extremes of the habitat and effectively reduce each individual contaminant present, not just the total contaminant load (COD). The primary aim of this investigation was to extend the previous study to a bioreactor run of 400 h, and by regular sampling, determine the temporal persistence of the inoculated strains and specifically the fate of the individual chemical components of the waste. Secondly, to compare the performance of inoculated bioreactors, in terms of pollution load reduction and consistent performance (reproducibility), with that of bioreactors with just the MWF indigenous community or with activated sludge, the current industrial norm.

Results

Chemical analyses

Four replicate bioreactors were established for each of the four treatments: bacterial consortium with, and in the absence, of the indigenous MWF community; indigenous MWF community; activated sludge from a municipal sewage works. Changes in pollution load were measured by chemical oxygen demand (COD), with an initial effluent COD value (time = 0 h) of approximately 48000 mg l^{-1}. Final mean COD reduction for the bacterial consortia inoculated bioreactors (with and without indigenous community present) was 83% ± 0.1% and 85% ± 0.4% respectively (Fig. 1). Mean COD reduction after 400 h in the bioreactors containing only the indigenous MWF community was 37% ± 1.5%. The bioreactors inoculated with activated sludge achieved a mean COD reduction of 38%, but showed greater variability in performance between replicated bioreactors (±12%). The fate of individual MWF components was determined by gas chromatography – mass spectroscopy (GC-MS). In bioreactors containing bacterial consortia, the majority of components (including the biocide) were no longer detected after a maximum of 300 h operation (Fig. 2). The only exceptions to this were benzotriazole and the lubrication agents, propylene glycol and amine propoxylate. However, all three of these components were degraded by >60% over 400 h bioreactor operation. In comparison, degradation of components in reactors containing MWF indigenous populations, with and without activated sludge communities, was poor. The majority of components (including the biocide) were reduced by <15% over 400 h operation. The only exceptions to this were benzotriazole (40 and 21% reduction), amine propoxylate (69 and 37%) and notably sebacic acid (43 and 100%) in reactors containing MWF indigenous populations with and without the activated sludge communities respectively.

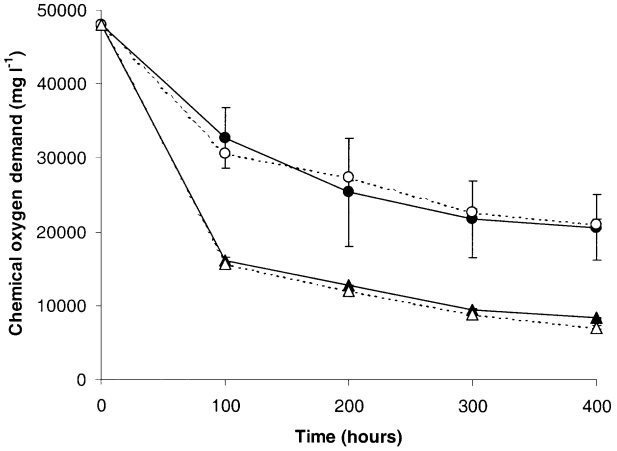

Fig. 1. Reduction of pollution load (chemical oxygen demand) in bioreactors over 400 h. Activated sludge with indigenous community (—●—); indigenous MWF community (—○—); bacterial consortium (—▲—); and bacterial consortium with the indigenous community (—△—). Error bars represent standard deviation of the mean (n = 4).

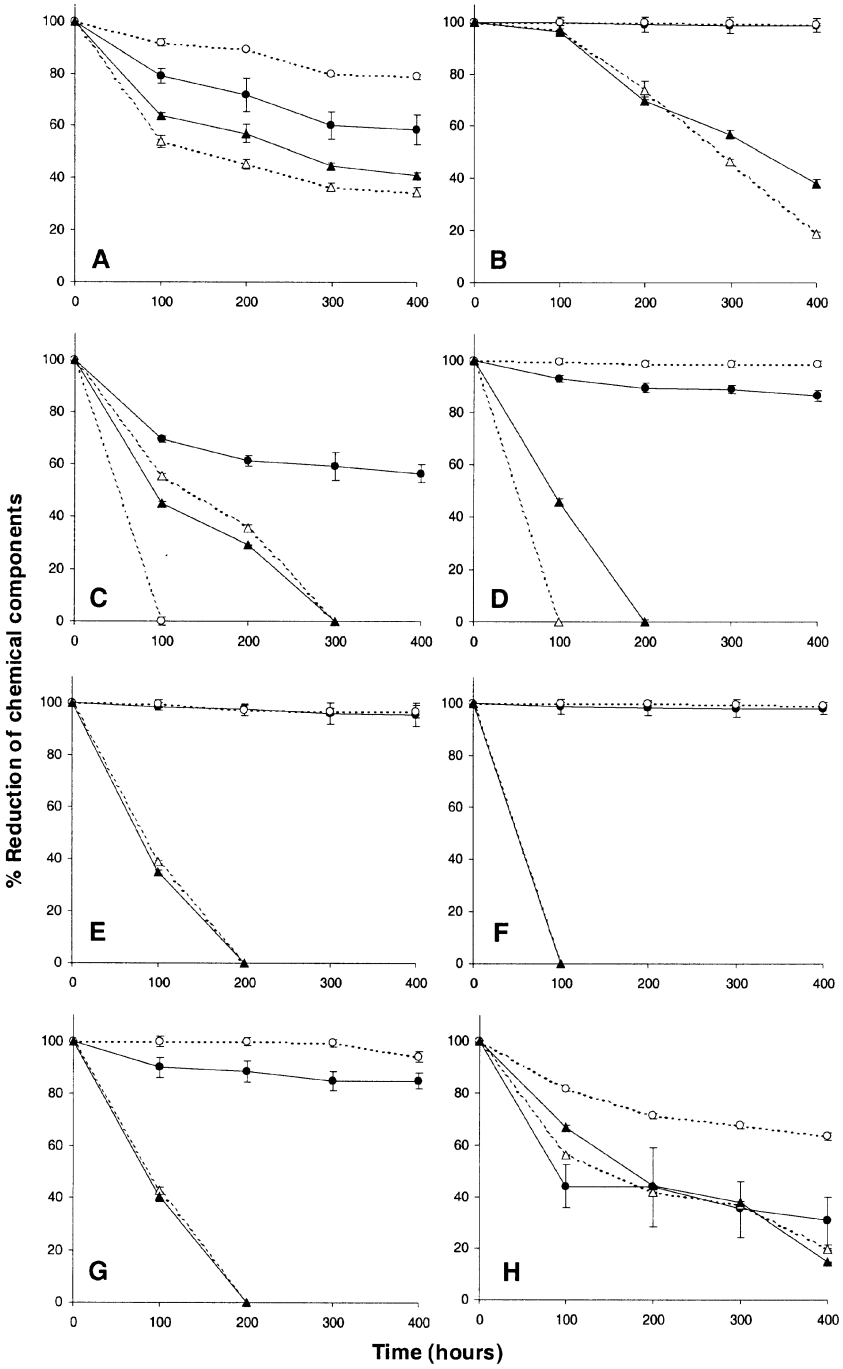

Fig. 2. Fate of MWF chemical components in bioreactors determined by GC-MS. Activated sludge with the indigenous MWF community (—●—); indigenous MWF community (—○—); bacterial consortium (—▲—); and bacterial consortium with indigenous MWF community (—△—). A, benzotriazole; B, propylene glycol; C, sebacic acid; D, lauric acid; E, dodecanedioic acid; F, glycerin; G, biocide; and H, amine propoxylate. Error bars represent standard deviation of the mean ($n = 4$).

Community structure and abundance determined by fluorescent in situ *hybridization*

Measures of the structure of the bacterial communities were determined by the analysis of data obtained with oligonucleotide probes of taxonomic resolution (class level) against independent DAPI counts. The probes, ALF1a, GAM 42a and HGC69a, were employed to detect bacteria belonging to the alpha and gamma subclasses of the *Proteobacteria*, and high G + C Gram-positive bacteria respectively (Fig. 3). Total DAPI counts in bacterial consortia reactors increased from approximately 3.3×10^6 to 1.9×10^7 cells ml^{-1} over the 400 h operation. Throughout the study period, community composition remained relatively constant, with little notable fluctuation, regardless of MWF indigenous community presence or absence, suggesting the persistence of members of the consortium (Fig. 3). For instance, counts from group spe-

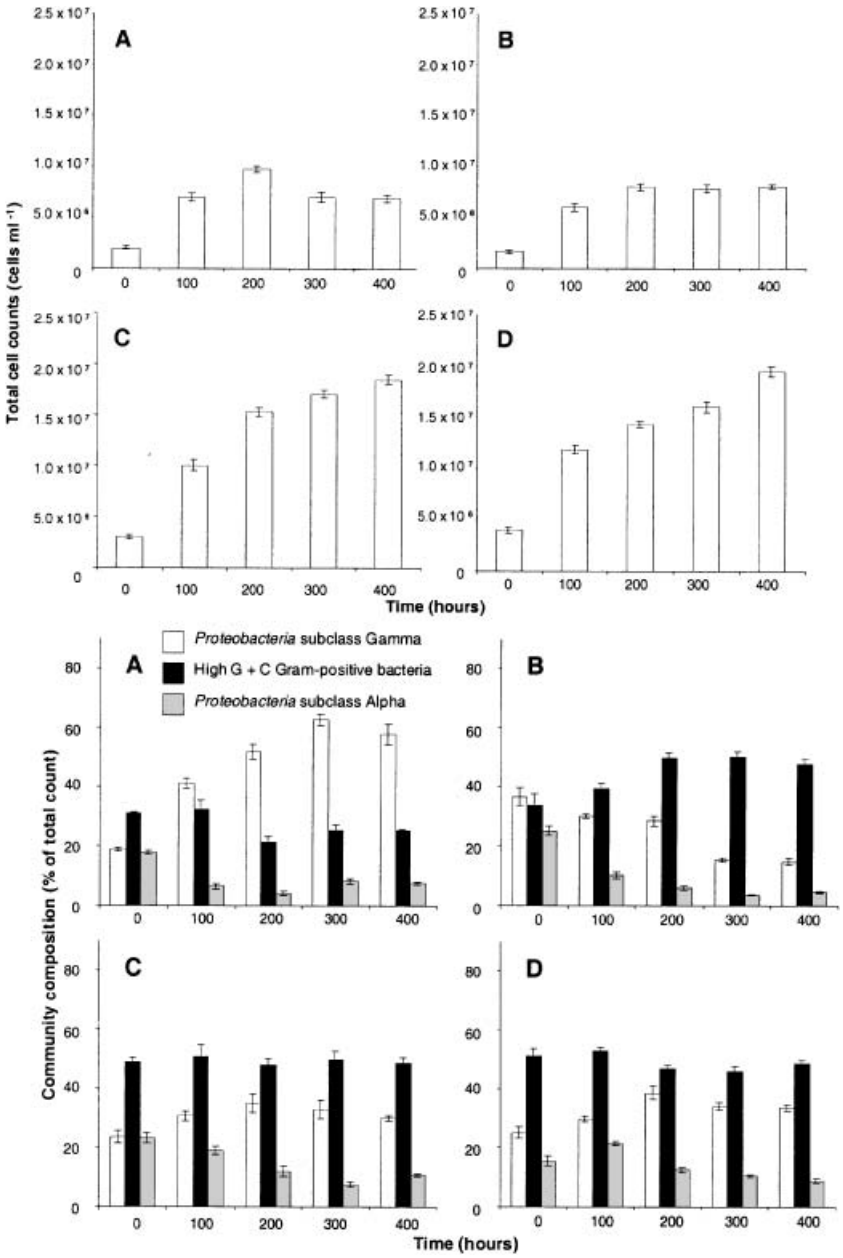

Fig. 3. Total cell counts obtained by DAPI staining and bacterial community structure determined by fluorescent *in situ* hybridization (% of total DAPI count) within reactors over 400 h.
A. Activated sludge with the indigenous MWF community.
B. Indigenous community.
C and D. Bacterial consortium without and with presence of the indigenous MWF community, respectively.
Community composition within bioreactors based upon the percentage of the total DAPI cells assigned to the HGC 69a (High G + C Gram-positive bacteria) and respective subclasses within the Proteobacteria by specific probes α 1b and γ 42 a. Error bars represent standard deviation of the mean ($n = 4$).

cific probes accounted for >90% of the total cells present. However, in reactors containing MWF indigenous populations, with and without activated sludge communities, community composition fluctuated throughout the study; the taxon specific probes accounting for 67–90% of the total cell counts.

Relationships between chemical parameters with bacterial abundance and community composition

When MWF chemistry composition (chemical oxygen demand and MWF component fate) were related to the total DAPI counts they were found to correlate significantly ($P < 0.05$) in the bacterial consortium bioreactors (with and without indigenous community presence), and to a lesser degree, bioreactors containing only indigenous MWF communities (Table 1). Bacterial counts exhibited linear inverse relationships with respect to chemical parameters, indicating a strong modulation of total cell count by the MWF pollution load (COD) and the chemical constituents. Essentially, as individual MWF constituents and chemical oxygen demand decreased, cell counts increased in response. In the bacterial consortia reactors, with MWF indigenous community present, significant relationships ($P < 0.05$) between bacterial abundance and all chemical parameters were observed, suggesting that the

Table 1. Relationships between total DAPI count and chemical parameters.

	Indigenous community		Bacterial consortium		Bacterial consortium and indigenous community	
	r	*P*	r	*P*	r	*P*
Chemical oxygen demand	0.974	0.005	0.939	0.018	0.973	0.005
Benzotriazole			0.978	0.004	0.965	0.008
Propylene glycol					0.946	0.015
Sebacic acid	0.950	0.013	0.978	0.004	0.922	0.028
Lauric acid	0.942	0.017	0.861	0.050	0.947	0.015
Dodecanedioic acid	0.898	0.039	0.978	0.004	0.971	0.006
Glycerol			0.861	0.050	0.970	0.006
Biocide			0.982	0.003	0.894	0.040
Amine propoxylate	0.975	0.005	0.981	0.003	0.828	0.038

All bacterial abundances that were significant ($P < 0.05$) exhibited linear inverse relationships with chemical parameters. Where r is correlation coefficient and *P* is significance at 95% level.

communities within those reactors could biodegrade all of the MWF components and effectively reduce the pollution load.

Relationships between culturable cell counts and MWF pollution load

Culturable cells (calculated as CFU ml^{-1}, percentage of the total DAPI count) in bacterial consortia containing reactors, with and without indigenous community presence, reached maximum mean values of 42 and 52%, respectively, at 100 h operation. In reactors containing only the MWF indigenous communities, cell culturability attained a maximum mean value of 24% at 200 h. In the activated sludge community bioreactors cell culturability was <1% throughout operation. When the relationships between cell culturability and percentage reduction of pollution load (chemical oxygen demand) per 100 h were compared, only bacterial consortia containing reactors showed a significant relationship ($P < 0.05$). Here cell culturability exhibited a direct linear relationship with COD reduction 100 per h (Fig. 4), suggesting the greatest reductions in MWF pollution load occurred when the bacterial consortium populations were at their most active.

Isolation and identification of bacterial isolates from bioreactor communities

Phenotypic community analysis by FAME (Table 2) revealed that the four members of the consortium became an established part of the bioreactor community to which they were introduced. Consortium members persisted in inoculated bioreactors even in the presence of the indigenous community, with the exception of *Bacillus sphaericus* (detected only at 0 h). Phenotypic diversity within activated sludge and indigenous community containing bioreactors was low, with between three and eight culturable species detected throughout bioreactor operation. The most abundant species detected in these bioreactors were *Clavibacter michiganensis*, *Alcaligenes xylosoxydans* and *Methylobacterium mesophilicum*.

Discussion

Exploitation of microorganisms in bioreactor based systems holds great promise for treating chemically mixed wastes, such as metal working fluids. However, in order

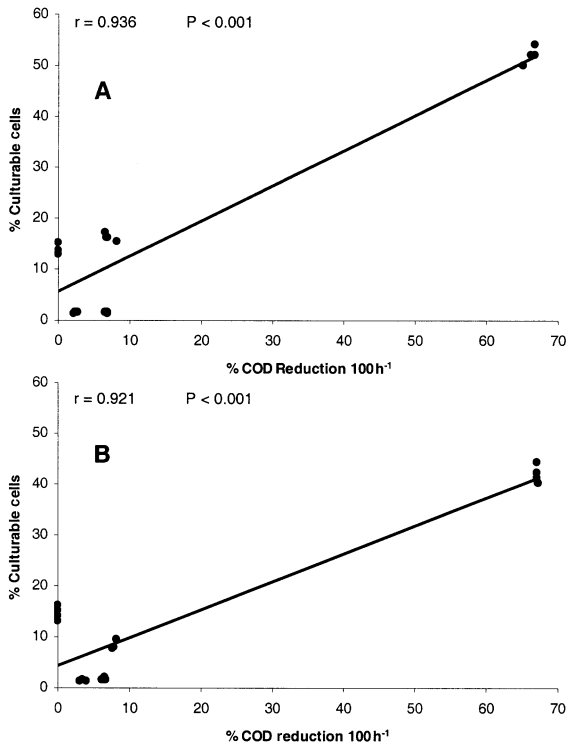

Fig. 4. Relationships between culturable cells and percentage reduction of pollution load (chemical oxygen demand) 100 h^{-1}, in (A) bacterial consortium and (B) bacterial consortium and MWF indigenous community bioreactors. Relationships presented are significant at $P < 0.05$. r = correlation coefficient.

Table 2. Frequency (%) per sample of bacterial taxa isolated from bioreactors over 400 h and identified by fatty acid methyl ester analysis ($n = 30$).

	Activated sludge and indigenous community					Indigenous community				
	0 h	100 h	200 h	300 h	400 h	0 h	100 h	200 h	300 h	400 h
Alcaligenes xylosoxydans		26.7	6.7	13.3		20.0	20.0	6.7	6.7	6.7
Bacillus pumilius						20.0	13.3	6.7		
B.sphaericus	20.0	6.7	20.0			33.3	33.3	6.7	6.7	
B.marinus						13.3		6.7		
B.oleronius	13.3									
Brevibacillus brevis	6.7	13.3								
Brevibacterium lyticum	6.7									
Brevundimonas diminuta					40.0		6.7	20.0	33.3	53.3
Cellulomonas flavigena		6.7	13.3	13.3	20.0					
Clavibacter michiganense	26.7	20.0	40.0	13.3	6.7	13.3	20.0	33.3	40.0	40.0
Curtobacterium flaccumfaciens		6.7	6.7							
Gordona rubropertinctus		6.7	0.0							
Methylobacterium mesophilicum	6.7	13.3	13.3	53.3	20.0		6.7	20.0	13.3	
Pseudomonas putida	13.3			6.7	13.3					
Rhodococcus erythropolis										
Stenotrophomonas maltophilia	6.7									

	Bacterial consortium					Bacterial consortium and indigenous community				
	0 h	100 h	200 h	300 h	400 h	0 h	100 h	200 h	300 h	400 h
Alcaligenes xylosoxydans										
Bacillus pumilius										
B. sphaericus						6.7				
B. marinus										
B. oleronius										
Brevibacillus brevis										
Brevibacterium lyticum										
Brevundimonas diminuta										
Cellulomonas flavigena										
Clavibacter michiganense	33.3	46.7	46.7	53.3	53.3	33.3	46.7	33.3	46.7	53.3
Curtobacterium flaccumfaciens										
Gordona rubropertinctus										
Methylobacterium mesophilicum	20.0	20.0	26.7	26.7	26.7	26.7	20.0	26.7	26.7	26.7
Pseudomonas putida	26.7	20.0	20.0	20.0	13.3	20.0	13.3	26.7	20.0	13.3
Rhodococcus erythropolis	20.0	13.3	6.7		6.7	13.3	20.0	13.3	6.7	6.7
Stenotrophomonas maltophilia										

for such approaches to be more universally accepted as a viable disposal technology, it is vital that the effectiveness and robustness of the approach is convincingly demonstrated. A key decision that must be made in order to establish efficient bioreactors is the nature and source of the microorganisms responsible for the degradative process. Although some samples of waste MWF contained high microbial counts (e.g. 10^7 cells ml^{-1}), others have low biomass and as such are not a reliable source of active degradative microbial populations. Furthermore, there is growing interest in applying a hybrid process whereby waste MWF are first ultra-filtered to remove the bulk of the COD load, and then passed to bioreactors to remove the remaining waste. However, ultrafiltration with membrane pore sizes ranging from 0.01 to 0.1 μm (Enviro-Wise, 1999), removes the microbial biomass and so it is necessary to re-inoculate the waste when it passes to the bioreactor. Because of these developing engineered processes, it is increasingly important to identify sources of bacteria that can maintain high population densities in bioreactors, catabolise the waste, be tolerant of its high toxicity and the extreme chemical fluctuations of the system. In a previous study we systematically assembled a mixed inoculum based on the selection criteria of their ubiquitous distribution, degradative ability and tolerance to the toxicity of co-contaminants (van der Gast *et al.*, 2002), and demonstrated their ability to persist in the waste and reduce the pollution load in a short-term study where samples were only taken at 100 h (van der Gast *et al.*, 2003). However, this study provided little indication of the longer-term performance (>100 h), reliability of the inoculum, or detailed assessments of the fate of the individual chemical components.

The results of this study confirm that the mixed inoculum, assembled on the basis of their natural abundance in MWF, degradative abilities and chemical tolerance, was very effective for bio-augmentation of bioreactors established for treating waste fluids. Despite the toxic nature of

the system (reflected by the <1% of culturable cells in reactors containing undefined communities from activated sludge), the introduced inoculum persisted, maintained a high viable biomass, and reduced not only the pollution load, but also all of the individual MWF components, including the biocide and the recalcitrant benzotriazole. The high culturability of consortia members in the MWF reflected the adapted nature of selected strains to the substrate. Based on FISH analyses alone, using group specific probes, it was difficult to determine the relative abundance of the bacterial consortium to that of MWF indigenous populations in the bacterial consortium inoculated bioreactors containing the indigenous community. However, judging from the strikingly close similarity in community structure between the bacterial consortium reactors, with (Fig. 3D) and without (Fig. 3C) the MWF indigenous community, we suggest that the bacterial consortium represented a significant component of the total community detected by FISH. Furthermore, *in situ* analyses using group specific probes when combined with phenotypic (FAME) assessment throughout the 400 h bioreactor operation demonstrated that members of the consortia represented a significant component of the total population detected in inoculated bioreactors. This supports the view that the introduced strains were highly competitive and suited to the extreme conditions of the MWF, certainly when compared to commonly used undefined communities from activated sludge, a common source of microorganisms in many similar industrial applications (Baker *et al*., 1983; Hamer, 1997). In addition, the reduction in the COD by the consortium was approximately 85% of the total pollution concentration: 30–40% more effective than the indigenous community alone and the reactors containing undefined populations from activated sludge. The assertion that the consortium members were well adapted to MWF is further supported by temporal measures following the fate of individual chemical constituents, by GC-MS. After 300 h, the majority of chemical constituents were undetectable in bioreactors containing the consortium. The exceptions to this were benzotriazole, amine propoxylate and propylene glycol, but even these were substantially reduced after 400 h bioreactor operation (>60%). Some constituents, most notably the biocide, propylene glycol, lauric acid, dodecanedoic acid proved to be particularly resistant to the degradative activity of the microbial community indigenous to MWF or from activated sludge.

In order to gain some insight into the functional role of the detected populations we correlated the pollution load (COD) and chemical composition with bacterial abundance and cell viability, detected within samples (Fig. 4). Significant ($P < 0.05$) relationships were only observed between cell viability and COD reduction in bacterial consortia containing reactors (with and without indigenous community presence). Cell culturability exhibited a direct linear relationship with COD reduction, this suggested that bacterial consortium members were active and responsible for the COD reductions observed within these bioreactors. This was confirmed when bacterial abundance was shown to exhibit linear inverse relationships with all chemical parameters (except with propylene glycol in consortia only reactors). This revealed that COD and MWF constituents declined as the bacterial consortia counts increased. The ability of the bacterial consortium to biodegrade the MWF components was particularly highlighted when their performance was compared to that of bioreactors containing indigenous MWF community, with and without activated sludge. A possible explanation for this poorer performance was the inability of these populations to biodegrade, or effectively resist the bacterial biocide and benzotriazole present in the MWF which has been shown to be toxic to microorganisms (van der Gast *et al*., 2002). A particularly encouraging aspect of the inoculated bioreactors was the consistent performance of the inoculum, an important feature in terms of scaled up applications on site. For instance, the performance of the inoculated bioreactors, in terms of COD load reduction (Fig. 1), showed less variation between the replicates than bioreactors containing indigenous communities or those from activated sludge. Furthermore, the performance in the inoculated bioreactors within 100 h was remarkably similar to previously reported results (van der Gast *et al*., 2003), highlighting consistent and reliable performance. Although these results are very encouraging, we are aware that the inoculum in the bioreactors was examined over only 400 h batch mode operation. We have now started extended bioreactor trials under semibatch mode operation to assess the long-term fate and performance of the inoculum.

Along with a few notable studies (Erb *et al*., 1997; Boon *et al*., 2000; Watanabe *et al*., 2002; He *et al*., 2003), this is one of the few reports in which it has been unequivocally demonstrated that bioaugmentation with selected strains, based upon an understanding of the diversity of indigenous populations in the target waste, can lead to more effective processing of wastes by bioaugmentation than applying uncharacterized communities. The combined application of *in situ* hybridization and culture dependent (FAME) techniques enabled us to follow the fate of the inoculum. The complementary techniques applied confirmed the predominance, persistent and highly adapted nature of selected strains and their ability to degrade each one of the chemical constituents of MWF. Furthermore, we are confident that the approach of linking information on the *in situ* microbial community composition with chemical analyses, will improve selection of effective inocula for other waste systems, and ultimately tailor consortia for bioaugmentation application. We

believe that this study provides convincing evidence that the failure of bioaugmentation, in many studies, is caused by poor selection of appropriate strains and limited understanding of the indigenous populations that naturally colonize these chemically complex and toxic fluids.

Experimental procedures

Metal-working fluid (MWF)

The MWF, used as the model effluent in this study, was an operationally exhausted synthetic fluid (Castrol Limited, UK), used as a coolant and lubricant in large scale continuous metal working processes, to machine tungsten carbide and steel. In brief, the fluid comprised of eight main chemical constituents including a formaldehyde-based biocide; benzotriazole (metal passivator); dodecanedioic acid, lauric acid and sebacic acid (corrosion inhibitors); amine propoxylate, glycerin and propylene glycol (lubrication agents). Fresh MWF is typically supplied as a concentrate, which is diluted with water to form a 6% v/v working fluid prior to use in machining operations. Because of issues of commercial sensitivity, formulation details are seldom divulged, especially in terms of the absolute concentrations of each of the MWF chemical constituents, as was the case in this study. What can be revealed is the total weight per volume of all components in the MWF which was approximately 43110 mg l^{-1}.

Inoculation conditions and bacterial consortia construction

Four inocula conditions were investigated: activated sludge from a municipal sewage works (Sandford-upon-Thames UK); indigenous MWF community (no added inocula); bacterial consortium [indigenous community removed from MWF effluent by filtration through 0.2 μm pore-size filters (Millipore, UK)]; bacterial consortium and MWF indigenous community. In brief the bacterial consortium was constructed by screening large numbers of waste MWF samples, and selecting the most spatially and temporally distributed strains with the best degradative performance (van der Gast *et al*., 2002). The consortium comprised of four bacterial strains: *Clavibacter michiganensis* (designated strain E863-G-1); *Rhodococcus erythropolis* (E863-AP-1.1); *Methylobacterium mesophilicum* (E865-LA-2); and *Pseudomonas putida* (AS-G-10). These four strains met all of the three selection criteria: (i) they were ubiquitous in spatially and temporally separate samples; (ii) could degrade the chemical constituents of MWF, and (iii) were tolerant to co-contaminants. The four strains were inoculated separately into 250 ml conical flasks containing 50 ml of tryptic soy broth (10% v/v Difco, UK) and fresh MWF concentrate (3% v/v). The individual cultures were incubated at 28°C, in an orbital shaker, for 12 h (cell counts = 10^7 cells ml^{-1}). The cell suspensions were removed and resuspended in MWF effluent, mixed together and added as a 10% v/v inoculum into the bioreactors. For the activated sludge bioreactors, cells were after centrifugation (3 g wet biomass) resuspended and inoculated as described above. The initial cell concentrations in the bioreactors (time = 0 h) for each condition were as follows: activated sludge and indigenous community, 2.0×10^6 ml^{-1}; indigenous community, 2.0×10^6 ml^{-1}; bacterial consortium, 3.0×10^6 ml^{-1}; bacterial consortium and indigenous community, 3.5×10^6 ml^{-1}.

Bioreactor operation

Biodegradation studies were performed in bubble column bioreactors (2 L total volume). For each inocula condition four replicate bioreactors were operated. All bioreactors were run under batch suspension conditions (400 h duration), using working volumes of 1.5 l. Air flow within the bioreactors was maintained at 200 l min^{-1}, using aquarium air pumps and air-spargers (Fisher Scientific, UK). Reactor temperature was maintained at 28°C ± 1°C, using water-heated jackets. Samples of 5 ml were taken for analyses at 50 h intervals over a 400-h period.

Pollution load measured by chemical oxygen demand (COD)

Reduction of pollution load in bioreactor samples was determined by colorimetric COD analysis using a LASA 100 mobile laboratory photometer (Dr Lange, UK) with COD cuvette test kits (range 5000–60 000 p.p.m., Dr Lange, UK). Metal-working fluid samples were prefiltered through 0.2 μm pore-size filters (Millipore, UK) and analyses performed according to the manufacturer's instructions.

Gas chromatography – mass spectroscopy (GC-MS)

Metal-working fluids were subjected to gas chromatography – mass spectroscopy (GC-MS) to detect presence of individual MWF components. The samples were prepared for injection by adjusting the pH to <2.5 with hydrochloric acid, the water component was allowed to evaporate and the concentrate resuspended in 1 ml of methanol (HPLC grade, Fisher Scientific, Lougborough, UK). Samples were methylated by adding 200 μl of resuspended concentrate to 50 μl trimethylsulfonium hydroxide solution (Fluka Chemike, UK). Individual compounds were separated on a HP 5890 Series 2 GC (Hewlett-Packard, UK) with a BPX5 column (5% phenyl equiv. polysilphenylene-siloxane, 25 m × 0.25 mm, Hewlett Packard, UK). Using a temperature programme of 40°C for 1 min, 7.5°C min^{-1} temperature ramp to 340°C and then held for 4 min. Separated compounds were analysed by electron bombardment using a HP MSD mass spectrometer (Hewlett Packard, UK). The resulting mass spectra were then analysed and individual components named by the HP Chemstation v 3.0 program (Hewlett-Packard, UK).

Fluorescent in situ *hybridization (FISH)*

Fluorescent *in situ* hybridization was performed as described previously (Whiteley and Bailey, 2000). The probes employed in this study were as follows: Alf1b and Gam42a, corresponding to the respective subclasses within the *Proteobacteria*; and HGC 69a specific to high G + C content Gram-positive bacteria. Group specific probe selection was based on the taxonomic groups the bacterial consortium members

belonged to, i.e. *P. putida* which is assigned to *Proteobacteria* subclass Gamma, *M. mesophilicum* (*Proteobacteria* subclass Alpha), *C. michiganensis* and *R. erythropolis* (both High G + C containing Gram-positive bacteria). All samples were counterstained with DAPI (4′, 6′-diamidino-2-phenylindole, Sigma chemicals, UK) for total cell enumeration with microscopy.

Phenotypic identification and characterization of bioreactor bacterial isolates

Bioreactor samples were serially diluted in phosphate buffer solution and spread plated onto R2A solid media (Oxoid, UK). The R2A plates were incubated for 24 h at 28°C. After incubation 30 isolates from each bioreactor condition were randomly selected from R2A plates containing 20–200 colonies. The phenotypic diversity and identification of individual strains isolated from bioreactor samples was determined by fatty acid methyl ester (FAME) analysis, as described previously (Thompson *et al*., 1993). The samples were injected into a Hewlett-Packard model 6890 series gas chromatograph and fatty acid peaks named using the Microbial Identification System (MIS) software (Microbial ID, Newark, DE, USA) and isolates identified using the MIS 'TSBA40 aerobe library' version 4 (1999).

Statistical treatment

In order to examine the relationships between community composition and the chemical composition of MWF in bioreactors, all data was analysed for significant correlations using the Minitab package (Minitab 12; Minitab). 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.

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