RESEARCH ARTICLE

Extended tracking of the microbial community structure and dynamics in an industrial synthetic metalworking fluid system

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Abstract

Understanding of the occupational exposure risk scenario and disease etiology associated with industrial metalworking fluids (MWFs) requires knowledge of the development and composition of their microbial diversity in relation to the underlying fluid management factors. In this study, a managed synthetic MWF operation freshly recharged following the dumping, cleaning, and recharge (DCR) process was tracked in real time for microbial community changes over a period of 1.25 years (65 weeks). The recharged fluid developed very high bacterial counts (viable and nonviable) fairly quickly after the DCR process, indicating its inadequacy. Genus-/group-specific real-time qPCR confirmed the prevalence of six potentially pathogenic/immunogenic microbial genera/groups, *viz.* pseudomonads, enterics, mycobacteria, legionellae, actinomycetes, and fungi. Selective culturing revealed *Acinetobacter* and *Bacillus* as the most frequently isolated Gram-negative and Gram-positive genera, respectively, in addition to the presence of fungi and actinomycetes. Endotoxin perturbations (< 1000 to $> 100\,000$ EU mL^{-1}) coincided with temporal increases in Gram-negative bacteria and/or periodic biocide additions. PCR-DGGE-sequencing revealed an expanded estimated bacterial richness (up to 23 bands per sample). Of the 16 dominant bacterial phylotypes identified, the majority were detected for the first time in MWF. Interestingly, the study revealed a crucial role for MWF brand, among other fluid factors, in modulating the community structure and dynamics.

Introduction

Metalworking fluids (MWFs) used in industrial machining and grinding operations may be oil or water based and semi-synthetic or synthetic formulations. While in use these water-based machining fluids support microbial growth, which introduces biological contaminants, including microbial cells or cell components and their related biological byproducts such as antigens and toxins. The fluid complexity is compounded by deliberate physico-chemical manipulations during operation and contamination with substances resulting from the manufacturing process such as tramp oil, hydraulic fluid, and particulate matter generated by grinding and machining operations.

Contaminated MWFs pose health hazards and may cause a variety of diseases in exposed machinists (Sandin *et al.*, 1990; Bruze *et al.*, 1995; Kreiss & Cox-Ganser, 1997; Rosenman, 2009). Microbial products (antigens, endotoxins, etc.) accumulated in contaminated MWF are presumed to be the etiological agents of inflammatory respiratory illnesses (Castellan *et al.*, 1987; Gordon, 1992; Thorne & DeKoster, 1996; Thorne, 2000; Gordon *et al.*, 2006; Thorne *et al.*, 2006). MWF-associated microbial species may vary in their immunogenicity (Gupta *et al.*, 2009), and the microbial antigen load may be contributed by both culturable (viable) and nonculturable (viable or nonviable) microorganisms (Mattsby-Baltzer *et al.*, 1989a; Lonon *et al.*, 1999; Gordon *et al.*, 2006). This emphasizes the need for understanding both culturable and nonculturable fractions as well as structure of the total microbial community in these fluids.

Considering the limitations of classical culturing approach to account for the viable but nonculturable (VBNC) cells and dead or nonviable (NV) cells that are

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present (Mattsby-Baltzer *et al.*, 1989b; Lange *et al.*, 1997), additional classical tools such as microscopy-based direct count method (Thorne *et al.*, 1996; Lange *et al.*, 1997; Selvaraju *et al.*, 2008a) have been adapted to estimate the total concentration of microorganisms in MWF. In addition, understanding the types of microorganisms colonizing these fluids is critical as certain MWF-associated microbial groups may produce potent antigens as demonstrated for *Mycobacterium immunogenum* in our studies (Gupta *et al.*, 2009). Modern DNA-based methods such as those custom developed in our recent efforts for these fluids can be employed to unambiguously detect and quantify specific microbial types in MWF without culture (Yadav *et al.*, 2003; Khan & Yadav, 2004b; Selvaraju *et al.*, 2005; Khan *et al.*, 2005; Selvaraju *et al.*, 2008b; Kapoor & Yadav, 2009). Culture-independent molecular methods such as denaturing gradient gel electrophoresis (DGGE) combined with amplicon sequencing known for analysis of the structure and dynamics of the total microbial community (Muyzer & Smalla, 1998) and identification up to the species level (Das *et al.*, 2007; Ibekwe *et al.*, 2007; Labbe *et al.*, 2007) in microbial ecological studies could be extended to these fluids.

The majority of the past studies on microbial diversity in the industrial machining fluids have been performed based on random batch sampling with little information on tracking (Mattsby-Baltzer *et al.*, 1989b; Lonon *et al.*, 1999; van der Gast *et al.*, 2003; Veillette *et al.*, 2004; Cyprowski *et al.*, 2007; Perkins & Angenent, 2010; Gilbert *et al.*, 2010; Murat *et al.*, 2012; Saha & Donofrio, 2012). The focus of the current study was therefore a real-time tracking of the microbial community dynamics and structure in an in-use MWF operation specially cleaned and recharged for the study. The tracking was performed for over a year (65 weeks) on this synthetic fluid operation in a large automotive parts manufacturing industrial setting. Furthermore, little is known on the role of fluid-related variables and fluid management practices in the development of microbial diversity in these fluids. The current temporal study therefore analyzed the role of such nonbiological fluid-related factors in the development and modulation of microbial community structure and dynamics.

In light of the foregoing background and rationale regarding the microbial community analyses in MWF, we specifically (1) quantified and compared the total microbial load of MWF by microscopy (light and epifluorescence) and culture; (2) quantified and compared the targeted microbial communities using a combination of selective culturing and group-/genus-specific real-time quantitative PCR coupled with DNA sequencing; (3) determined global microbial community structure and dynamics using a DGGE approach combined with DNA sequencing; and (4) studied endotoxin buildup dynamics in the MWF operation. The experimental data were interpreted in conjunction with the nonbiological fluid parameters periodically recorded and provided by the plant management. Part of the data from this study was presented at the 106th annual conference of the American Society for Microbiology (Selvaraju *et al.*, 2006).

Materials and methods

Fluid sampling

The synthetic metalworking fluid tracked in this study was sampled during an MWF operation in an automobile parts manufacturing plant in the northern part of the United States. In preparation for this microbial tracking study, the MWF operation was subjected to the commonly practiced dumping, cleaning, and recharge (DCR) process before the sampling was initiated; the operation was continued over the entire sampling period using routine fluid management practices, such as periodic addition of biocide and fresh fluid ('top-loading'), fluid change, and other activities, as conventionally employed in that plant like other industrial settings of this nature. One-liter samples were periodically drawn from the test synthetic fluid operation (main pit) beginning the day of pit recharge (designated as week 0). A total of 50 samples were drawn over a period of 65 weeks (1.25 years; Table 1). Samples 1 through 37 were received consecutively every week except for a temporary 2-week shutdown of the plant between samples 14 and 15. Beginning at sample 38, periodic samples were received fortnightly (biweekly). Synthetic MWF brand A was used in the operation for the first 25 weeks (samples 1 through 24); subsequently, it was top-loaded with another brand of synthetic MWF (brand B), at week 26 (sample 25). The pit was again top-loaded with fresh fluid of the same brand (brand B) at week 44 (sample 40); the samples 25 through 40 therefore contained a mixture of both the synthetic fluid brands, and samples 40 through 50 contained predominantly one synthetic fluid (brand B). Temporal information on various physical/chemical parameters of the operation such as fluid dilution, fresh fluid addition, fluid pH, oil content, and biocide addition along with biostrip information (to estimate culturable microbial load) on the fluid samples was provided by the source plant (Supporting Information, Table S1).

Microscopic and culturable counts of microbial communities

Twenty-milliliter aliquots of the MWF samples were centrifuged at 23 400 ***g*** for 30 min, and the pellet was resuspended in 1 mL normal saline. Serial dilutions of the

Table 1. Group-/genus-specific primers used for real-time PCR in this study

Microbial groups/genera	Primer sequence	Reference
Eubacteria (all bacteria)	F: 5′ ACT CCT ACG GGA GGC AGC AGC 3′ R: 5′ GTA TTA CCG CGG CTG CTG GCA C 3′)	Walter *et al.* (2000)
Pseudomonads	F: 5′ GAG TTT GAT CCT GGC TCA G 3′ R: 5′ CCT TCC TCC CAA CTT 3′	Johnsen *et al.* (1999)
Enteric bacteria	F: 5′ TCC BGTG AAA AGG GCA CTA AC 3′ R: 5′ CAG ATC GGA CAT CAA ATA GC 3′	Bayardelle & Zafarullah (2002)
Mycobacteria	F: 5′ CTG GTC AAG GAA GGT CTG GC 3′ R: 5′ GAT GAC ACC CYTC GTT GCC AAC 3′	Khan & Yadav (2004a)
Legionellae	F: 5′ AGG GTT GAT AGG TTA AGA GC 3′ R: 5′ CCA ACA GCT AGT TGA CAT CG 3′	Wellinghausen *et al.* (2001)
Actinomycetes	F: 5′ GGA TGA GCC CGC GGC CTA 3′ R: 5′ CGG CCG CGG CTG CTG GCA CGT A 3′	Heuer *et al.* (1997)
Fungi	F: 5′ GGC TCT CGC ATC GAT GAA GAA C 3′ R: 5′ CTT TTC CTC CGC TTA TTG ATA TGC 3′	White *et al.* (1990)

F, forward primer; R, reverse primer.

resulting pelleted cell suspension were used to determine bacterial numbers by microscopy and culturing.

Microscopy

Microscopic counting was performed using Reich counting slides (Bellco Glass, Inc, Vineland, NJ) based on modification of the breed counting technique (Vincent, 1918). The smear was prepared by spreading a defined amount (5 μL) of the appropriately diluted cell suspension in the marked circle (with an interior area of 1 cm^2) on the slide. Total bacterial count was determined by light microscopy using methylene blue staining (0.3% solution). Viable and nonviable bacterial cell counts were determined by epifluorescence microscopy (Nikon Eclipse 50i) using Baclight Live/Dead viability staining kit (Molecular Probes, Eugene, OR). Ten different microscopic fields were counted for each type of microscopic count [*viz.*, total, viable, and nonviable count (NV)], and the cell counts mL^{-1} were calculated according to the following formula: cell count (cells mL^{-1}) = $N_b \times M_f \times 1/V \times 1/C$, where N_b = average number of cells/field, M_f (microscopic field) = number of fields cm^{-2} area of the slide, V = volume of stained cell suspension applied to the slide, C = concentration factor (fold concentration of original sample in relation to the staining sample suspension).

Culture

Appropriate serial dilutions from the microbial pellet were spread (100 μL each) on tryptic soy agar (TSA) and incubated at 37 °C for 48 h to assess total culturable counts expressed as colony-forming units (CFU) per mL of the original fluid.

VBNC count

Difference between the total viable count (epifluorescence microscopy based) and the total culturable count (agar culture based) yielded the VBNC count.

Targeted community analysis

Selective culturing and DNA sequencing-based identification of specific genera/species

Individual MWF samples were plated on the general medium (TSA) and several selective agar media for the isolation of specific (targeted) microbial genera/groups. The microbial species thus obtained were identified by DNA sequencing. Briefly, 1 mL cell suspensions in normal saline prepared from 20-mL MWF aliquots by centrifugation as above were analyzed for targeted genera/groups by spread agar plating (100 μL) using the following selective/specific agar media and appropriate incubation conditions: pseudomonas isolation agar (PIA; pseudomonads; 25 °C), Middlebrook 7H10 agar (MBA; mycobacteria, 37 °C + 5% CO_2), Sabourauds dextrose agar (SDA; fungi; 30 °C), actinomycetes isolation agar (AIA; actinomycetes; 30 °C), eosin methylene blue agar (EMB; enteric bacteria, 37 °C), and buffered charcoal yeast extract agar with antibiotics (BCYE; legionellae, 37 °C + 5% CO_2). These media were all DIFCO brand purchased from Difco Laboratories, Detroit, MI, or Becton, Dickinson & Co., Sparks, MD.

Morphologically distinct colonies growing on the specific agar media were individually identified by PCR and DNA sequencing using amplification primers (Table 1) based on the 16S rRNA V2-V3 variable region for bacteria and the 18S–28S internal transcribed spacer region for

the fungi. Briefly, the individual isolates were cultured in an appropriate broth, and the genomic DNA was extracted from a 1.5-mL culture pellet using the method described earlier (Khan & Yadav, 2004b). PCR mixture (50 μL) consisted of 200 μM dNTPs, 100 ng of each of the forward and reverse primers, 5 μL of the 10x buffer, 2.5 U of Pfu Ultra high fidelity DNA polymerase (Stratagene, Cedar Creek, TX), and 100 ng of the isolated DNA. PCR amplification program for bacterial isolates involved initial melting cycle (95 °C for 2 min) followed by 30 amplification cycles of 95 °C for 30 s (denaturation), 56 °C for 30 s (annealing) and 68 °C for 1 min (extension), and a final extension at 68 °C for 5 min. For fungal isolates, the PCR program included initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min (denaturation), 55 °C for 1 min (annealing), and 72 °C for 2 min (extension). The PCR products were purified using Montage PCR filter units (Millipore Corporation, Billerica, MA) and sequenced at the University DNA core facility.

Real-time PCR-based quantification of the specific genera/groups

We first developed a qPCR protocol (using the available specific primers) for each of the targeted genera/groups: pseudomonads, enteric bacteria, *Mycobacteria, Legionellae*, *Actinomycetes*, and fungi. Standard curves for qPCR amplification were generated using the group-/genus-specific primers (Table 1) and template DNA from representative test strains from the individual groups, *viz.* *Pseudomonas fluorescens* ATCC 13525 ($r^2 = 0.98$, slope = −2.89, efficiency = 122%), *Escherichia coli*-DH5α ($r^2 = 0.99$, slope = −3.73, efficiency = 85.4%), *M. immunogenum* ATCC 700506 ($r^2 = 0.99$, slope = −2.85, efficiency = 124%), *Legionella pneumophila* spp. *pneumophila* ATCC 33215 ($r^2 = 0.99$, slope = −3.95, efficiency = 78.5%), *Streptomyces griseus* ATCC 11746 ($r^2 = 0.99$, slope = −3.19, efficiency = 105.82%), and *Phaenerochaete chrysosporium* ATCC 24725 ($r^2 = 0.99$, slope = −3.44, efficiency = 95.3%). Because the standard curves were prepared by spiking the test strains in the MWF matrix to emulate the real sample scenario, the observed deviation in the PCR efficiency from the ideal range could be due to the inherent inhibitory constituents in MWF matrix. For direct real-time PCR quantification in the fluids, cell pellet obtained from 100-mL sample aliquot was subjected to DNA extraction using the method described earlier (Khan & Yadav, 2004b) followed by amplification with Hotstart Taq DNA polymerase and the compatible SYBR Green PCR master mix (Stratagene and Takara Bio Inc., Madison, WI) using a Smart Cycler (Cepheid, Sunnyvale, CA). Reaction mixture (20 μL) consisted of 10 μL of 2x master mix, 40 ng of each of the forward and reverse primers, and 50 ng of the template DNA. PCR amplification program for pseudomonads involved initial melting cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation), 50 °C for 15 s (annealing), and 72 °C for 30 s (extension). PCR conditions for the other genera/groups of organisms remained the same except for the annealing temperature, *viz.* 60 °C (enteric bacteria), 58 °C (mycobacteria), 57 °C (legionellae), 63 °C (actinomycetes), and 55 °C (fungi).

PCR-DGGE and DNA sequencing-based analysis of whole community structure and dynamics

Total microbial DNA was extracted from the fluid samples (100 mL aliquots) containing the mixed microbial community as described above (Khan & Yadav, 2004b). The V2-V3 region of 16S rRNA gene was amplified using the following universal primer pair with a GC clamp at 5′-end of the forward primer: HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) (McBain *et al.*, 2003). A 100-ng aliquot of the extracted microbial community DNA was used for PCR reaction using Ex-Taq DNA polymerase kit (Takara Bio Inc.) in a final reaction (50 μL) containing 200 μM dNTPs, 100 ng of each primer, 1.25 U of Taq polymerase, and 2.5 μL of BSA. The DGGE analysis was carried out using a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). Samples were loaded onto a 12% polyacrylamide gel with a denaturation gradient ranging from 30 to 50% (100% denaturing solution contained 40% formamide (v/v) and 7 M urea) in 1x TAE electrophoresis buffer. Each gel included a marker lane. The reference marker was developed by extraction and amplification of prominent bands from the DGGE gel. Electrophoresis was carried out initially at 50 V for 15 min followed by 200 V for 5 h at 60 °C. Gels stained for 10 min with ethidium bromide (0.5 μg mL^{-1}) were imaged using Kodak Gel Documentation 1D image analysis software version 3.5 (Rochester, NY). The banding patterns corresponding to the individual samples were analyzed using GELCOMPAR II (version 4.5) software (Applied Math, Keistraat, Belgium) and clustered based on Dice/UPGMA (Das *et al.*, 2007).

To characterize the individual bands on the DGGE gel, gel slices were carefully excised and processed for sequencing (Ibekwe *et al.*, 2007). The extracted DNA from individual bands was purified and sequenced. Sequence similarity/identity search using BLAST revealed the bacterial phylotypes corresponding with the DGGE bands.

Endotoxin analysis in MWF

Endotoxin levels in MWF samples were determined using the Chromogenic Limulus Amebocyte Lysate (LAL) assay kit (Cambrex, Walkersville, MD). MWF supernatant obtained after centrifugation (23 400 ***g*** for 30 min) was serially diluted with endotoxin-free water. Equal volumes (50 μL each) of the LAL reagent (Cambrex) and the diluted samples were mixed and incubated at 37 °C for 10 min. A 100-μL volume of the chromogenic substrate was added and incubated for 6 min. The reaction was stopped by adding 25% acetic acid. The absorbance was read at 405 nm using a Wallac Victor2 plate reader (Perkin Elmer, Boston, MA). The endotoxin concentration was determined from the standard curve generated using the endotoxin standard provided in the kit (Cambrex).

Results and discussion

Microbial community dynamics (viable, nonviable, and culturable fractions)

In the 65-week tracked MWF system, total bacterial counts reached very high levels for the majority of the samples, falling in the range of 10^7–10^{10} CFU mL^{-1}, although a transient expansion in the range was observed in the middle phase (samples 27 through 37) of the study (Fig. 1). The bacterial concentration observed is close to the range noted in previous reports on industrial MWF systems (Virji *et al.*, 2000; Veillette *et al.*, 2004; Gilbert *et al.*, 2010) and is reminiscent of microbial loads in fermentation broths and gut/sewage samples (Quigley & Quera, 2006; Martinez-Nieto *et al.*, 2011). There was a substantial microbial load (9.18×10^6 cells mL^{-1}) even in the first sample drawn right after the DCR process at week 0, indicating that the DCR of the pit did not help eliminate the microbial contaminants. The residual microbiota such as in the pipes that were inaccessible to cleaning as well as that associated with the surfaces/crevices/biofilms seemed to have seeded the system (Foxall Vanaken *et al.*, 1986; Veillette *et al.*, 2004).

Total cell counts maintained a steady level during the first 19 weeks (Fig. 1). Frequent addition of fresh fluid (at weeks 3, 8, and 11) or biocide Kathon 886 MW (at weeks 7, 11, and 16; Table S1; Fig. 1) simultaneously provided fresh nutrients (for growth) or antimicrobial compounds (for restricting bacterial multiplication), respectively, thereby preventing any net change in the viable fraction. This was confirmed by the observation that the slow net increase in the total cell counts observed during the initial sampling period was mainly due to the nonviable fraction, whereas the other two fractions, viable and VBNC, remained almost constant (Fig. 1). The slow increase, if any, in viable fraction in the initial 19 weeks also implied that gradual adaptation of the microbiota to the MWF fluid type used is also evidenced by a higher microbial buildup in subsequent weeks.

There was a nonlinear trend in the bacterial community changes during the later part of the sampling period in terms of total, viable, and nonviable fractions (Fig. 1). This was possibly due to the dilution effect caused by the addition of a very large amount of fresh fluid (7571 liters

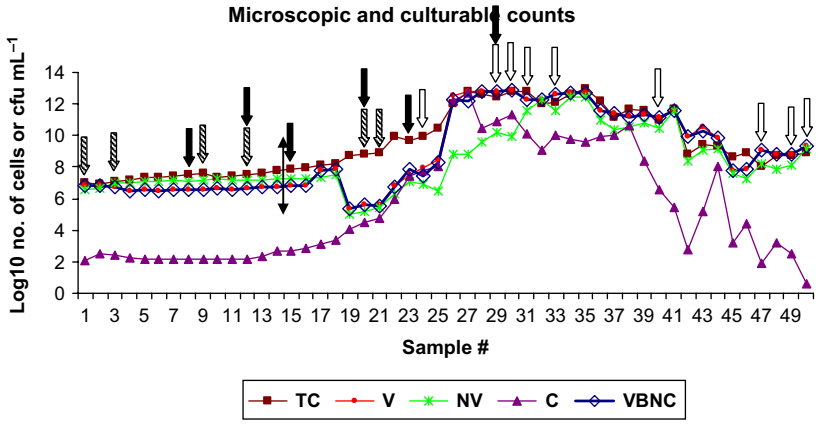

Fig. 1. Microbial community dynamics in a synthetic MWF operation tracked for 1.25 year based on microscopic and cultural analysis. The sampling schedule spanned a 65-week period drawing samples at weekly or biweekly intervals as described under the Materials and methods section. TC, total count; V, viable count; NV, nonviable count; C, culturable count; VBNC, viable but nonculturable count. The bacterial numbers are expressed as counts mL^{-1} [for TC, V, NV, and VBNC] or CFU mL^{-1} [for C]. Nonbiological information on fluid-related parameters is indicated by vertical arrows as follows. Striped arrow (⇛): addition of fluid brand A; unfilled arrow (⇨): addition of fluid brand B; filled black arrow (➡): addition of biocide (Kathon or Grotan); double thin arrow (↔): 2-week temporary shutdown of the operation after week 13 of operation. The details on these and other fluid-related parameters are provided in Table S1.

equivalent to 2000 US gallons) in the beginning of this sampling period (week 40) and intermittent fresh fluid additions during the remaining period (Table S1). The culturable fraction showed increases mainly coinciding with the fresh fluid additions, particularly coinciding with the change in fluid type from brand A to brand B in the 25th week of operation. This suggested that the new fluid, which had a different composition, was relatively more conducive to the multiplication of the MWF microbiota. In general, the culturable counts were significantly lower compared to the total counts (TC). In the last phase of the study, the culturable counts showed an erratic trend with alternate increases and decreases and reached minimum, almost undetectable, levels (4.0 cells mL^{-1}) at week 65. These data collectively indicated that majority of the MWF microbiota existed in noncultivable form, and therefore, doing a culture alone would grossly underestimate the microbial load of the MWF, as also concluded in previous field MWF studies by us and others (Yadav *et al.*, 2003; Veillette *et al.*, 2004; Selvaraju *et al.*, 2008a, b).

The biocide addition regime practiced at the plant did not show any immediate net reductive effect on the total bacterial count, indicating a largely bacteriostatic (rather than bactericidal) effect of the resulting biocide–MWF mixture in the in-use fluid. Interestingly, biocide addition (Table S1) was always associated with an immediate or subsequent increase in the total oil content (and tramp oil content in most but not all instances). Such increase in oil content coincided with an increase in total cell counts at week 16 (coinciding with sample 15). Hence, a more informed biocide regime may therefore be required to control the microbial contamination comprising the diverse bacterial phylotypes. This warrants the development of (1) novel broad-spectrum biocides or optimization of the biocide regime using the existing commercial biocides; and (2) a test inoculum for biocide efficacy testing based on predominant MWF-colonizing microbial species to enable the selection of more effective biocides for MWF applications.

Taken together, the results implied a more apparent impact of the fluid-related (such as change in fluid brand or fluid dilution) as compared to process-related factors (such as biocide addition) on the microbial levels in MWF. Among these parameters, a change in the fluid brand caused the most dramatic shift in the microbial community levels and structure. In general, no clear association was observed between the community shift and changes in other nonbiological parameters such as pH and tramp oil in the sampled synthetic MWF system. In contrast to this study, a noticeable effect of increasing the fluid pH and decreasing the leaking tramp oil in controlling the microbial levels was reported in an earlier study on mineral oil-based soluble MWF (Virji *et al.*, 2000). The different nature of the fluid types between the two studies could be the underlying reason for the observed difference.

Targeted community analysis

Selective culturing

Use of the general medium (TSA) for bacterial culturing yielded four different genera identified by DNA sequencing as *Acinetobacter*, *Bacillus*, *Stenotrophomonas*, and *Paenibacillus* (Table 2). The commercial selective media meant to isolate the targeted groups, *viz.* pseudomonads (PIA), enterics (EMB), mycobacteria (MBA), legionellae (BCYE), and actinomycetes (AIA), either did not yield the intended genus/group at all or yielded nontargeted genera/groups in addition to the targeted genera/groups (Table 2). This emphasized that these commercial presumably selective media are not absolutely selective. In the event of relevant detection, the selective culturing approach detected the targeted groups in some but not all 50 samples (Table 2). For instance, PIA did not yield any *Pseudomonas* (or any other bacterial colonies) in the first 29 weeks of sampling (samples 1 through 28). However, typical pseudomonad colonies were detected in several of the subsequent samples, albeit at low levels, *viz.* 7.5×10^0 CFU mL^{-1} (sample 29), 4.59×10^2 CFU mL^{-1} (sample 30), and 1.0×10^0 CFU mL^{-1}–3.3×10^2 CFU mL^{-1} (samples 32 through 37, 42, and 43). Samples 32 and 33 also yielded colonies of another bacterial species *Stenotrophomonas maltophilia* on PIA. Likewise, EMB agar yielded nonenteric isolates up to sample 23 and a *Pseudomonas* sp. in sample 24. MBA yielded no mycobacteria, but a *Streptomyces* sp. from sample 6 and three other genera (*Alcaligenes faecalis*, *Paenibacillus* sp., and *Acinetobacter* sp.) from later stages of sampling. AIA yielded fungal (*Fusarium*) colonies from samples 1 through 9 and typical bacterial colony types from samples 25 and 26 (*Paenibacillus* sp.) and 32 (*Bacillus* sp.). Similarly, BCYE yielded non-*Legionella* isolates and SDA did not yield any fungal colonies from the first 30 samples. However, samples 31 through 36 and 43 through 47 grew fungal colonies albeit at extremely low levels. These were identified as species of *Fusarium*, *Cladosporium*, and *Nectria* by sequencing of the 18S–28S ITS region (Table 2).

Overall, the culturable bacterial community consisted mainly of Gram-negative bacteria, with *Acinetobacter* being the most frequently isolated genus. An earlier report had suggested an initial colonizer role for this genus in laboratory studies (Salmeen *et al.*, 1987). In contrast, a majority of previous studies have highlighted predominance of other Gram-negative genera, particularly

Table 2. Microbial (bacterial and fungal) isolates from MWF obtained using various culture media

Culture medium*	Isolate ID	BLAST analysis details			
		'Best match' similarity to (type strain)	Accession number of the 'best match' sequence	Base pairs used for comparison	% similarity
TSA	*Acinetobacter seohaensis*	95% (SW-100)	DQ518593	207	97
	Acinetobacter sp.	NA	JQ754327	226	100
	Bacillus cereus†	99% (ATCC 14579)	AY296806	206	99
	Paenibacillus glucanolyticus†	99% (NBRC 15330 T)	JX860237	197	99
	Stenotrophomonas sp.	NA	JX500581	225	100
EMB	*Acinetobacter* sp.		JN860345	206	99
	Bacillus fusiformis†	99% (ATCC 7055)	AF478083	201	99
	Bacillus sphaericus†	99% (ATCC 14577)	AF478090	205	99
	Brevibacillus agri†	99% (NBRC 15538)	KF031530	233	100
	Chryseobacterium sp.	NA	HF678400	201	99
	Enterococcus casseliflavus†	99% (NBRC 100478)	HF936967	201	99
	Pseudomonas stutzeri	99% (ATCC 17588)	KC796794	200	99
	Streptomyces sp.‡	NA	D44303	121	99
PIA	*Pseudomonas* sp.		JQ316361	200	99
	Stenotrophomonas maltophilia	99% (NBRC 14161)	KC689303	204	99
	Stenotrophomonas sp.	NA	EU301687	224	100
MBA	*Acinetobacter* sp.	NA	JQ754327	211	99
	Alcaligenes faecalis	99% (NBRC 13111)	FJ226423	206	99
	Bacillus cereus†	100% (ATCC 14579)	GQ487537	198	100
	Bacillus sp.†	NA	JF900053	235	99
	Enterococcus casseliflavus†	98% (NBRC 100478)	HF936967	200	100
	Pseudomonas stutzeri	99% (ATCC 17588)	HM135448	205	99
	Streptomyces sp.‡	NA	D44303	121	99
BCYE	*Acinetobacter radioresistens*	99% (NBRC 102413)	HE651925	206	99
	Acinetobacter sp.	NA	JQ754327	203	100
	Stenotrophomonas maltophilia	99% (NBRC 14161)	KC593548	126	87
AIA	*Paenibacillus glucanolyticus*†	99% (NBRC 15330 T)	JX860237	202	99
	Bacillus pumilus	98% (ATCC 7061)	KF158229	276	98
	Fusarium sp.§	NA	JX290168	345	99
SDA	*Fusarium oxysporum*§	98% (ATCC MYA 4623)	HF546394	352	99
	Cladosporium sp.§	NA	GU395509	347	99
	Nectria haematococca§	98% (ATCC MYA 4622)	AF178411	336	99

NA means not available. As the BLAST analysis did not yield the species name, no 'type strain' could be used for comparison.

*Full names of the culture media are provided under the Materials and methods section. Their composition was obtained from standard microbiological media manuals.

†Gram-positive isolates (the unmarked isolates are Gram-negative).

‡Actinomycete isolates.

§Fungal isolates.

Pseudomonas (Mattsby-Baltzer *et al.*, 1989b; Lonon *et al.*, 1999; van der Gast *et al.*, 2003; Gilbert *et al.*, 2010). Few studies have reported predominance of other non-*Pseudomonas* genera, namely *Klebsiella pneumoniae* (Chazal, 1995), *Pantoea agglomerans/Citrobacter freundii* (van der Gast *et al.*, 2003), *Shewanella putrefaciens* (Cyprowski *et al.*, 2007), and *Ochrobactrum* spp. (Gilbert *et al.*, 2010). Enteric bacteria, such as *Alcaligenes faecalis* which may be opportunistic pathogens, were also cultured from the test operation in the current study. Interestingly, this species was the most predominant Gram-negative organism in MWF in a recent study (Perkins & Angenent, 2010). Multiple genera of Gram-positive bacteria and fungi were isolated. In this context, our study disagreed with the existing reports which suggest that the Gram-positive bacteria are usually not present in microbially contaminated MWFs (Kreiss & Cox-Ganser, 1997) and most likely are contaminants from the work environment or workers' microbiota (Veillette *et al.*, 2004). Furthermore, it has been suggested that fungal species may be found in systems following a permanent or temporary die-off of pioneering bacterial species (Rossmore, 1985), although this was not observed in the current study, which also contrasted with a recent French study (Murat *et al.*, 2012) that related the presence of Gram-positive bacteria in automotive industry fluids with a mineral oil-based rather

than a synthetic MWF composition and further showed a correlation between the occurrence of Gram-positive bacteria and *M. immunogenum*.

Genus-/group-specific real-time qPCR

This analysis revealed the presence of all six targeted genera/groups and allowed the detection of even the difficult-to-culture genera *Mycobacterium* and *Legionella*. However, considering the range of observed PCR efficiency values of the standard curves (possibly due to inhibitory effect of the MWF matrix), the results on changes in target groups are semi-quantitative. For better interpretation of the qPCR-based dynamic changes, the results in the 50 MWF samples in terms of individual target groups/genera, *viz.* pseudomonads, enterics, mycobacteria, legionellae, actinomycetes, and fungi, were divided into four phases (Fig. 2), namely phase 1 (samples 1 through 18), phase 2 (samples 19 through 28), phase 3 (samples 29 through 37), and phase 4 (samples 38 through 50). Nevertheless, detailed samplewise perturbation profiles for individual targeted genera/groups are also presented (see Fig. S1). Taken together, temporal changes in population showed initial low and steady levels for each targeted group except fungi which showed evidence of multiplication (phase 1). All targeted groups then showed a sudden surge in their numbers beginning at phase 2 (sample 19), the reason for which is not quite clear. Phase 2 indicated multiplication and/or peak levels of the individual bacterial genera/groups except enterics. While all groups declined in phase 3 and almost disappeared in phase 4, enteric group showed steady levels and dominated in the last phase (Fig. 2). The analysis therefore revealed that only certain genera/groups (pseudomonads, enterics, and fungi) underwent clear temporal increases indicating their colonizing ability, whereas other groups (*Mycobacteria, Legionellae, Actinomycetes*) seemed to transiently appear and disappear as contaminants during the operation.

Representative qPCR amplicon sequencing for the targeted groups mycobacteria and legionellae identified the organisms at the genus and/or species level. Analysis of the sequenced *Mycobacterium*-specific *hsp* gene amplicons from phase 2 (samples 19 through 26) using BLAST revealed maximum similarity (maximum identity: 85–86%, maximum score: 368–483) with *M. madagascariense* (Kazda *et al.*, 1992). Other species of *Mycobacterium* associated with MWF have been implicated in cases of hypersensitivity pneumonitis in machine workers (Kreiss & Cox-Ganser, 1997; Wallace *et al.*, 2002; Beckette *et al.*, 2005; Trout *et al.*, 2003). The sequence analysis of *Legionella*-specific PCR product derived from the DNA extract of the initial samples revealed closest match to an unspeciated *Legionella* (maximum score = 712) followed by

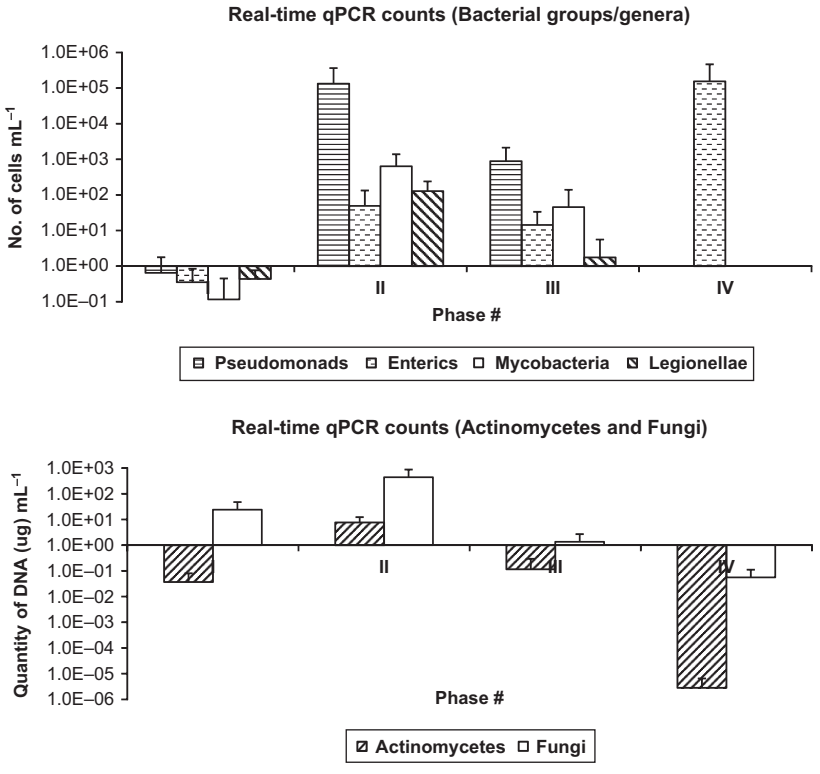

Fig. 2. Real-time qPCR-based estimates of the targeted microbial groups/genera. Quantitative levels for bacterial groups/genera are expressed as counts mL^{-1} (upper panel); the levels of filamentous organisms including actinomycetes and fungi were assessed based on the amount of genomic DNA expressed as $\mu g\ mL^{-1}$ (lower panel). The values are plotted as mean ± SD of triplicates for each sample. The *x*-axis represents arbitrary phases of the sampling period (phase 1 through phase 4). Phase 1 includes samples 1 through 18; phase 2 includes samples 19 through 28; phase 3 includes samples 29 through 37; and phase 4 includes samples 38 through 50.

L. quanteirensis (sequence similarity score = 690). Considering that the species of *Mycobacterium* and *Legionella* detected by PCR in this study could not be isolated by culture, but represent unconventional species not yet reported from MWFs, further confirmation is warranted on their ability to colonize MWF and their prevalence in field MWF. DNA sequencing of the qPCR amplicons for other groups revealed the presence of an unidentifiable species of *Pseudomonas* and species of multiple enteric genera (*Shigella* sp., *E. coli*, and *Salmonella* sp.).

Inconsistencies in the levels of enteric bacteria and other specific groups detected by selective culturing versus qPCR emphasized that DNA-based methods are more reliable for sensitive and specific detection of microbial contamination in MWF, a phenomenon observed for pseudomonads and mycobacteria in earlier MWF studies from our laboratory and other groups (Yadav *et al.*, 2003; Khan & Yadav, 2004b; Selvaraju *et al.*, 2008b; Veillette *et al.*, 2008; Kapoor & Yadav, 2009; Saha & Donofrio, 2012).

Global community structure analysis (DGGE-DNA sequencing)

Whole bacterial community structure showed temporal variations in the test fluid operation. In all, 10–23 different bands per sample were discernible across the study. GelCompar analysis of the DGGE banding patterns revealed that the 50 samples are groupable into 12 clusters (Fig. 3). Cluster 1 included samples 1 through 10, which showed a microbial community consisting of 15 different bacterial phylotypes. Cluster 2 included samples 11 and 12, which differed from cluster 1 by the absence of 1 and appearance of 2 new bands leading to a difference of 3% in the banding pattern. Cluster 3 included samples 13 and 14, which differed from cluster 2 by 2%. Samples 15 through 18 were grouped into cluster 4 and showed a banding pattern different from that of cluster 3 by 5%. Cluster 5 included samples 19 and 20, which showed a difference of 1% in the banding pattern of previous samples. Samples 21 through 24 were grouped into two separate clusters (samples 21 and 24 in cluster 6; samples 22 and 23 in cluster 7), which differed from each other by 2.5%. Taken together, the microbial community in MWF samples 1 through 24 was highly conserved. Samples 25 and 27 were grouped into cluster 8, which showed a banding pattern differing from that of samples 26 and 28 by 2.5% and 1%, respectively. A major community shift was seen in sample 29, which was grouped with sample 30 in cluster 9 and differed from cluster 8 by 11.5%. Samples 32 through 40 were included in cluster 10 showing a banding pattern different from that of cluster 9 by 12%. Sample 31 showed a unique banding pattern differing from cluster 9 and 10 by 3% and 8.5%, respectively. Cluster 11 included samples 42 through 44, which differed from cluster 10 by 1%. Sample 41 showed a banding pattern differing from cluster 11 by 9%, but similar to samples 45 through 49 and was grouped with these samples in cluster 12. Sample 50 showed a unique banding pattern consisting of 21 different bacterial phylotypes.

Sequencing of the individual amplicon bands from the DGGE gel lanes helped to identify a total of 16 different bacterial genera (Table 3). Notably, a *Mycobacterium* sp. was detected among the identified bacteria. The commonly reported genus *Pseudomonas* yielded an additional band implying the presence of more than one species of this genus. Nonetheless, majority of the 16 genera identified in this study are reported for the first time in contaminated MWF.

The DGGE results implied that the microbial community in the industrial synthetic fluid system tracked had a more complex structure (10–23 different bacterial phylotypes) than previously described. For instance, this is in contrast to the earlier findings, where phenotypic and genotypic methods revealed that the microbial communities in industrial spent MWFs had low diversity (van der Gast *et al.*, 2002, 2003; Gilbert *et al.*, 2010; Murat *et al.*, 2012). The microbial community structure was found to be highly conserved in the first 24 samples (the banding pattern differing from one another by 1–5%). This reflects the adaptation of the microbial community to the fluid type (brand A) that was used for 25 weeks, indicating a highly selective nature of the MWF substrate (van der Gast *et al.*, 2003). The change in fluid type from brand A to brand B at week 26, however, led to a major community shift as apparent from sample 29. This could be due to the preferential multiplication and/or selection of different bacterial phylotypes in the two fluid brands, again consistent with the highly selective nature of the substrate (which varies from one MWF brand to another).

Endotoxin buildup and dynamics

Endotoxins, the heat-stable lipopolysaccharide–protein complexes contained in the cell envelopes of all Gram-negative bacterial species, are released from cells generally as a result of death of the cell, or the lysis or disruption of the integrity of the outer membrane/cell wall structure (Galanos *et al.*, 1979). The freshly recharged fluid sample received right after DCR at week 0 (sample 1) showed a substantially high threshold level (2483.97 EU mL^{-1}) of endotoxin (Fig. 4). The endotoxin levels varied widely between the initial (samples 1–29) and the advanced (samples 30–50) stages of the operation, albeit with surges

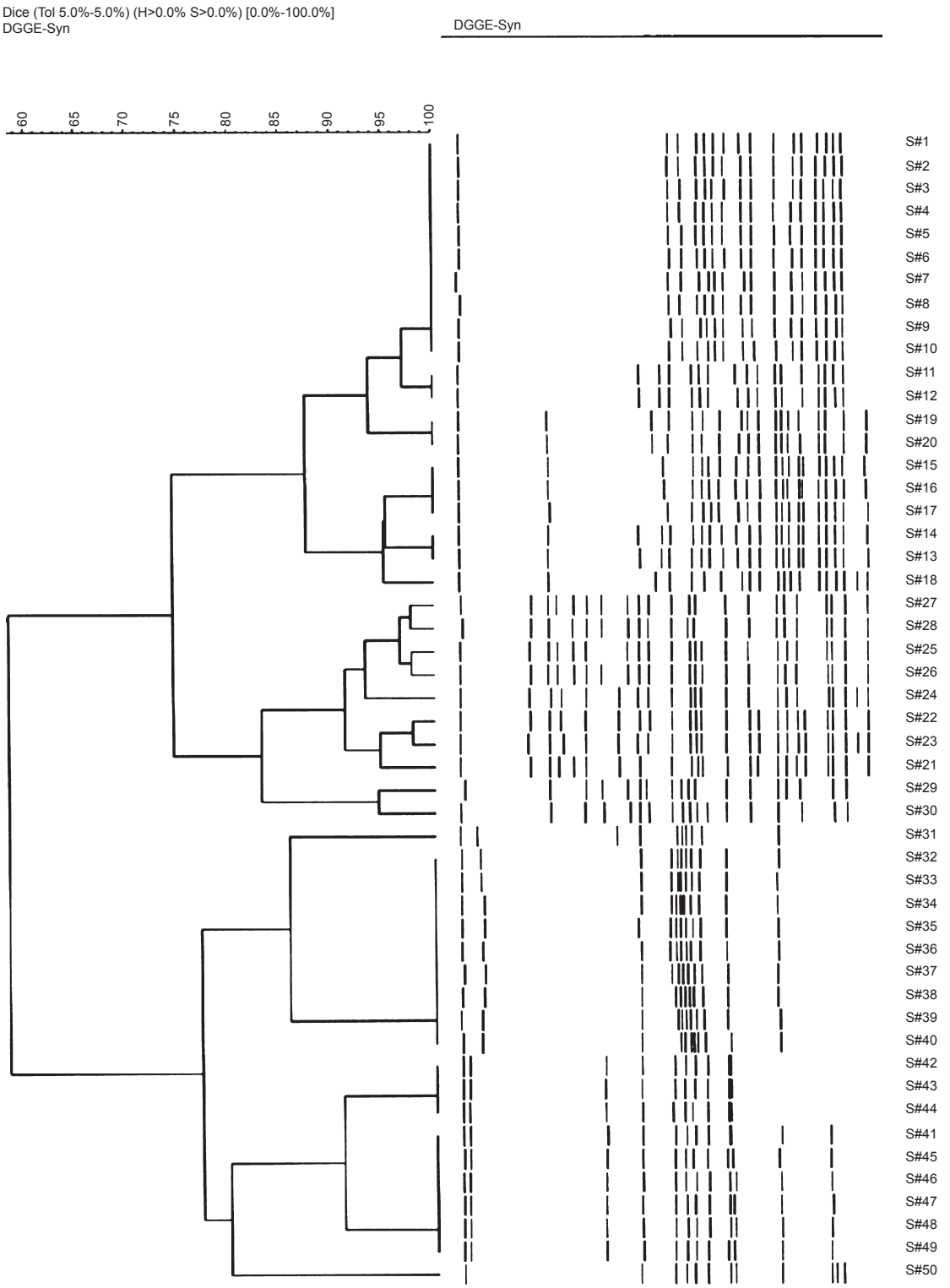

Fig. 3. Dice/UPGMA cluster analysis of DGGE fingerprints representing bacterial community structure of metalworking fluid samples. The samples were grouped into 12 clusters. A band tolerance of 5% was used. Only dominant bands from MWF samples 18 through 26 included in this comparison were sequenced and bacterial identities determined as presented in Table 3. For example, identity of the bands in sample # 18 (from top to bottom in that order) in this figure coincided with the following band numbering (and bacterial phylotypes) in Table 3: B16, B13, B2, B15, B14, B1, unnamed band, B3, B4, B5, unnamed band, B9, B10, B12, B16, B11, B7, B8, unnamed band, and B17.

Table 3. Bacterial species identified based on PCR-DGGE coupled with DNA sequencing analysis of the microbial community in in-use synthetic MWF

		BLAST analysis details			
DGGE gel band(s)	Bacterial ID (sequence based)	'Best match' similarity to (type strain)	Accession number of the 'best match' sequence	Base pairs used for comparison	% similarity to the 'best match', %
B1	*Pseudomonas* sp.	NA	DQ225137	164	89
B2	*Pseudomonas pseudoalcaligenes*	100% (ATCC 17440)	NR_037000	141	100
B3	*Acinetobacter towneri*	95% (CCUG 50769)	NR_028849	167	93
B4	*Sinorhizobium fredii*	99% (UDSA 205)	NR_102919	62	97
B5	*Methylobacterium extorquens*	89% (IAM 12631)	NR_074138	123	89
B6	*Rhodococcus* sp.	NA	EF067838	68	93
B7	*Brucella melitensis*	97% (ATCC 23456)	NR_074149	87	97
B8	*Arthrobacter phenanthrenivorans*	94% (Sphe3)	NR_074770	146	95
B9	*Comamonas testosteroni*	88% (ATCC 11996)	NR_029161	163	88
B10 and B15	*Azospirillum irakense*	100% (ATCC 51182)	NR_044949	69 and 61	100
B11	*Sinorhizobium fredii*	99% (UDSA 205)	NR_102919	138	97
B12	*Pseudoxanthobacter soli*	100% (DSM 19599)	NR_044225	141	91
B13	*Rhizobium tropici*	94% (CFN299)	NR_102511	35	94
B14	*Methylobacterium radiotolerans*	92% (JCM 2831)	NR_036824	143	93
B16	*Methylobacillus flagellatum*	100% (strain K)	NR_074178	70	96
B17	*Beijerinckia mobilis*	99% (ATCC 35011)	NR_042180	90	94

NA, not available; ID, identification.

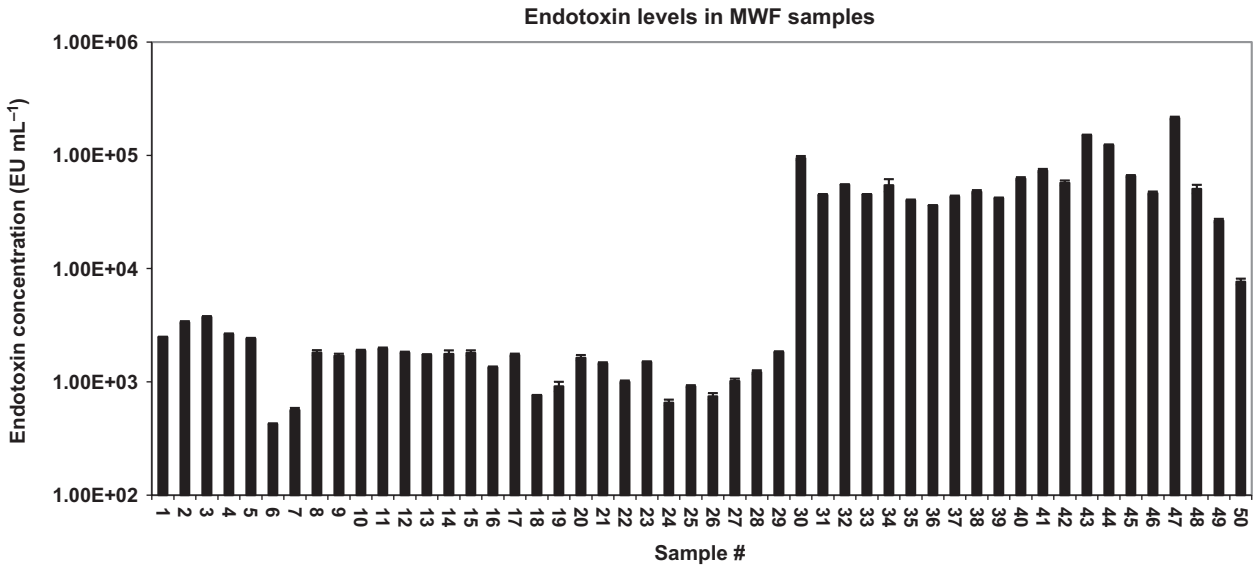

Fig. 4. Endotoxin levels in the temporally drawn samples from the synthetic MWF operation, as determined by LAL assay. The values plotted represent mean ± SD of triplicates for each sample and are expressed as endotoxin units per mL (EU mL^{-1}). The sampling schedule spanned a 65-week period as described under the Materials and methods section.

during each of the two stages. Taken together, there were two broad ranges of endotoxin level, 425–2644 EU mL^{-1} in the first 30 weeks and 7677–213276 EU mL^{-1} in the last 35 weeks. The surge in the endotoxin levels at two points, one at week 7 and another at week 31 (Fig. 4), coincided with the biocide additions at weeks 7 and 30 (Table S1), respectively, presumably because of biocide-mediated cellular damage and release of endotoxin from the Gram-negative bacterial fraction of the microbiota. The surge in the total cell counts at week 27 did not cause a corresponding surge in endotoxin levels. Likewise, fluctuations in the viable count did not coincide with the fluctuations in endotoxin levels; however, perturbations in the NV coincided with endotoxin fluctuations.

Collectively, these observations further supported the previous presumption that bacterial cell death-induced cell wall disintegration led to the release of their lipopolysaccharide component into the surrounding medium, and contributed to the observed increase in endotoxin level. As no biocide additions were made after week 31, the surge in the endotoxin levels at the two subsequent points, one in sample 43 and another in sample 47, appeared to be the result of the observed increase in the Gram-negative fraction, particularly enteric bacteria, of the bacterial community. Earlier studies also indicated the correlation between levels of Gram-negative bacteria and endotoxins (Mattsby-Baltzer *et al.*, 1989a; Milton *et al.*, 1990). Taken together, measurement of endotoxin levels can be used as a marker to determine the load of Gram-negative bacterial community in the fluid.

In conclusion, the industrial MWF systems, although periodically subjected to DCR with an intent to get rid of the microbial contamination, readily get seeded back again by the residual microbiota retained in the DCR-inaccessible areas and surfaces of the system. The sampled MWF system contained very high counts of bacteria, majority of which were, however, present in nonculturable form and hence were not detected by conventional culturing even using the commercially available selective media. Our results show that the fluid management practices prevalent in these industrial operations such as top-loading of fresh fluid and fluid dilution provide a refreshed microenvironment for the growth of microorganisms. On the other hand, periodic biocide additions may lead to a buildup of nonviable and/or VBNC bacterial cell counts in these fluids and particularly facilitate bacterial lysis leading to endotoxin buildup. Microbial community in the studied operation was comprised of diverse microbial genera, the nature of which dramatically fluctuated with the MWF formulation (brand) used. Use of molecular approaches in this study revealed several new bacterial colonizers, hitherto unidentified in MWF, emphasizing that a more diverse bacterial community than previously reported can colonize these fluids. Importantly, genera of the human pathogen groups normally associated with water sources particularly enterics were detected and found to show temporal increase in these fluids. Saprophytic mycobacteria and legionellae were detected in certain samples, but were not culturable or predominant in this operation. Therefore, the DNA-based methods now available for MWF analysis should be used for routine temporal monitoring of microbial diversity as well as reliable detection and identification of potentially pathogenic genera/groups in these complex matrices. This analytic practice will aid in designing timely and effective strategies/measures for the control of microbial growth and allow the assessment of exposure risk scenario and prevention of infections/outbreaks in metalworkers occupationally exposed to these fluids.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Real-time qPCR-based accumulation levels of the targeted microbial groups/genera in individual samples.
Table S1. Non-biological information from the source plant on the synthetic metalworking fluid operation.