Assessment of bacteria and archaea in metalworking fluids using massive parallel 16S rRNA gene tag sequencing

N. Di Maiuta¹, A. Rufenacht² and P. Küenzi²

¹ microsTECH AG, Olten, Switzerland
² Blaser Swisslube AG, Hasle-Rüegsau, Switzerland

Significance and Impact of the Study: Water-miscible metalworking fluids provide a suitable base of life for micro-organisms, mainly bacteria and fungi. Earlier publications suggested that the diversity is rather low, but these studies were largely based on heterotrophic plate counts. This might have resulted in underestimation of population density and microbial diversity as some organisms might just refuse to grow. This study used high-throughput sequencing in the absence and presence of propidium monoazide to explore bacterial and archaeal presence in metalworking fluids. We established that diversity is low and bacterial populations are dominated by the genus Pseudomonas spp.

Keywords:
archaea, bacteria, diversity, fungi, metalworking fluids, microbial contamination, microbiocides, PCR, propidium monoazide.

Abstract

Determination of the bacterial diversity in industry-based liquid in-use water-miscible metalworking fluid (MWF) samples was targeted by massive parallel multiplex DNA sequencing, either directly or upon pretreatment with propidium monoazide (PMA) that allows differentiation between intact and physically damaged cells. As MWFs provide a suitable basis of life for micro-organisms, the majority is preserved by biocides. ‘Bio-concept’ fluids on the other hand are bactericide free, which intentionally leads to substantial bacterial populations. Samples from both fluid types were chosen: A median of 51 operational taxonomic units at genera level (OTUs) were detected per sample, but only 13 were present at or above 15% of the total population in any PMA-treated sample analysed. As both fluid types were mainly dominated by Pseudomonas spp., we resolved this genus on the species level and found the Pseudomonas oleovorans/pseudoalcaligenes group to predominate. We also looked for archaea and detected Methanobrevibacter spp., albeit in <3% of all samples analysed.

Introduction

Water-miscible metalworking fluids (MWFs) are widely used in metal manufacturing industries. Formulation concentrates containing 5 to >15 organic ingredients are mixed with water at ratios typically ranging from 3% (v/v) to 15% (v/v), depending on the end-use application. The high water content and the evenly mixed in chemical components may provide a suitable base of life for micro-organisms, thereby representing a major risk for microbial-derived consequences on fluid quality, integrity and performance (Theaker and Thompson 2010), as well as being a possible cause for adverse health effects (Simpson et al. 2003). Micro-organisms in the environment are highly diverse, including organisms from three domains: Bacteria, archaea and eukarya, but until now, only bacteria and fungi had been recovered from these fluids (Passman 2006; Trafny 2013). Thus, biocides are often incorporated into MWF formulations.

Biocides used as preservatives were suspected to create increased dermatitis risks for operators (Maier et al. 2009) and their usage has been restricted due to recently adopted regulations. Consequently, some manufacturers abstain from using high-profile bactericides and rather rely on growth-inhibiting factors such as alkanolamines (Rossmore 1993). Others focus on bactericide-free MWFs that accept a substantial population of a waterborne bacterial species belonging to the Pseudomonas oleovorans/
outgrowth of the pseudoalcaligenes group, typically in the range between \(10^3\) to \(10^8\) CFU per ml. These so-called ‘bio-concept’ products are thought to exert a selection pressure mainly allowing the growth of this bacterial group (Dilger et al. 2005). What exactly exerts this selection pressure is unknown and diverse biologically active molecules have been suspected such as low concentrations of fungicides that may confer a selective advantage to \textit{P. oleovorans}/

\textit{pseudoalcaligenes}. Similar effects have been shown in other settings by using sublethal doses of antibiotics (Anderson and Hughes 2014).

Micro-organisms in MWFs include both aerobic and (facultative) anaerobic bacterial genera such as \textit{Acinetobacter} spp., \textit{Pseudomonas} spp., \textit{Shewanella} spp., \textit{Stenotrophomonas} spp., \textit{Comamonas} spp., \textit{Morganella} spp., \textit{Citrobacter} spp., \textit{Alcaligenes} spp., \textit{Micrococcus} spp., \textit{Staphylococcus} spp., \textit{Streptococcus} spp., \textit{Bacillus} spp. and \textit{Mycobacterium} spp. as well as numerous species of fungi such as \textit{Fusarium} spp., \textit{Exophiala} spp., \textit{Trichoderma} spp. and \textit{Penicillium} spp. (Trafny 2013; Kapoor et al. 2014). \textit{Pseudomonas} spp. is arguably the most common genus in MWFs and described as Gram-negative, facultative anaerobic Gammaproteobacteria, ubiquitously occurring in environmental sources such as soil and water. Notably, some publications suggested that the diversity in MWFs is actually rather low (van der Gast et al. 2003; Gilbert et al. 2010).

However, studies on functionality of biocides/biostatics as well as the ‘bio-concept’ were largely based on cultivation of micro-organisms on nutrient-rich media to demonstrate the performance of these systems. This method is also widely applied to identify micro-organisms and has disadvantages, as the majority of micro-organisms do not grow on artificial media (Epstein 2013). Traditionally, tryptic soy agar plates with subsequent specification based on growth attributes are applied, supplemented by the biochemical determination of specific properties or analysis of protein patterns by MALDI-TOF (Rezzonico et al. 2010). Alternatives are the use of denaturing gradient gel electrophoresis combined with amplicon sequencing and specific real-time PCR (Kapoor et al. 2014), and the testing of biomass using adenosine triphosphate as indicator (Passman and Kűienzi 2015). This study aimed at characterizing the bacterial diversity at genus level in mineral oil-based, preserved and ‘bio-concept’ fluids by application of massive parallel 16S rRNA gene tag sequencing. In addition, we applied PMA pretreatment to exclude physically damaged cells (Nocker et al. 2007). This allowed a more accurate and wider picture of the colonization of MWFs. We also investigated the presence of archaea, which are widespread in soils, water and animals and have also been detected in indoor environments (Pakpour et al. 2016).

Out of the scope of this investigation were biofilms in coolant systems and machines. However, a recent study suggested that neither the number of bacteria nor the size of microbial populations were affected by the presence or absence of biocides in industrial biofilm samples and several species of \textit{Enterobacteriaceae} were identified to be the main surface colonizers in their investigation (Trafny et al. 2015).

**Results and discussion**

First, the 16S rRNA gene was targeted to allow identification of OTUs in 179 mineral oil-based MWF samples. Eighty-five per cent of the detected OTUs were present at relative abundances below 0.1% in any sample and were subsequently excluded from further analyses. In general, a median of 56 OTUs was detected (52 in ‘bio-concept’ and 57 in preserved fluids). To exclude physically damaged cells, a subset of 62 samples (47 ‘bio-concept’ and 15 preserved) was additionally treated with PMA (Nocker et al. 2007), which slightly reduced the median number of detectable OTUs to 51 in total (50 in ‘bio-concept’ and 56 in preserved fluids). Only \textit{Acinetobacter} spp. and \textit{Pseudomonas} spp. were detectable in 100% of PMA-treated samples with a median abundance of 0.14 and 90.11% in ‘bio-concept’ and 0.51 and 36.59% in preserved fluids respectively (Table 1). \textit{Pseudomonas} spp. was the dominating OTU present in all samples and this dominance was more pronounced in PMA-treated ‘bio-concept’ fluids (Fig. 1a), whereas in preserved specimen no comparable shift was observable (Fig. 1b). Shown are the 10 OTUs with the highest median abundance in either preserved or ‘bio-concept’ samples: Most of these OTUs were thus present in both fluid types in top 10 positions. Shifts between untreated and treated populations were significant based on a paired \(t\)-test for \textit{Pseudomonas} spp. (‘bio-concept’: \(t_{\text{stat}} = 7.207, \ t_{\text{crit}} = 2.023, \ P < 0.001; \) preserved: \(t_{\text{stat}} = 3.909, \ t_{\text{crit}} = 2.145, \ P < 0.001\)). We propose that \textit{Pseudomonas} spp. gain most easily access to MWFs through the water supply, and then propagate comfortably in the bactericide-free environment of ‘bio-concept’ fluids, whereas preservation generally represses growth, thus provoking a more limited, but undirected diversity. Population development here likely depends on type and concentration of the active substance as well as on the environment. Table 1 shows frequency, median and relative abundance of these OTUs. These data were used to calculate a modified Jaccard Index (Yue and Clayton 2005) comparing these PMA-treated ‘bio-concept’ and preserved model populations resulting in a value of 0.535 (53.5% similarity).
Table 1 Operational taxonomic units with the highest median abundance in either 'bio-concept' or preserved samples and the formula for the modified Jaccard index (Yue and Clayton 2005) used to calculate the similarity between these 'bio-concept' and preserved model fluids treated with propidium monoazide (53.5% similarity)

| 'Bio-concept'       | Median abundance (%) | Relative abundance (%) | Preserved       | Frequency (%) | Median abundance (%) | Relative abundance (%) |
|---------------------|----------------------|------------------------|-----------------|---------------|----------------------|------------------------|
| Acinetobacter spp.  | 100                  | 0.14                   | 0.46            | 100           | 0.51                 | 4.13                   |
| Blastomonas spp.    | 72                   | 0.04                   | 0.63            | 93            | 0.28                 | 2.40                   |
| Citrobacter spp.    | 72                   | 0.04                   | 0.63            | 93            | 0.48                 | 1.93                   |
| Dysgonomonas spp.   | 70                   | 0.34                   | 0.91            | 93            | 0.48                 | 1.93                   |
| Morganella spp.     | 83                   | 0.66                   | 1.77            | 100           | 1.55                 | 9.30                   |
| Mycobacterium spp.  | 72                   | 0.91                   | 1.10            | 93            | 2.03                 | 9.21                   |
| Propionibacterium spp. | 76             | 0.03                   | 0.10            | 93            | 0.20                 | 0.29                   |
| Pseudochrobactrum spp. | 72           | 0.50                   | 0.53            | 93            | 0.84                 | 0.93                   |
| Pseudomonas spp.    | 100                  | 0.11                   | 83.87           | 100           | 36.59                | 44.83                  |
| Shewanella spp.     | 74                   | 0.13                   | 0.75            | 93            | 0.24                 | 0.32                   |
| Sphingomonas spp.   | 67                   | 0.03                   | 2.71            | 79            | 0.68                 | 10.91                  |
| Wautersiella spp.   | 76                   | 1.18                   | 1.39            | 93            | 1.55                 | 1.94                   |
| Others              | 100                  | 2.82                   | 5.48            | 100           | 6.55                 | 13.22                  |

\[
\sum_{i} f_{p,g,q} = 0.535.
\]

That populations in 'bio-concept' and preserved fluids are similar, but not necessarily identical, became obvious when populations from different samples, but from a single product each, were compared. As Pseudomonas spp. substantially dominated the 'bio-concept' fluids, microbial populations in these samples are highly similar to each other and to the 'bio-concept' model population in Table 1 (modified Jaccard Index of 0.996) although these samples were not pretreated with PMA. On the other hand, bacterial populations in preserved products were often less similar to each other, even if Pseudomonas spp. remained the main OTU in the majority of samples (Fig. 2). Here, the similarity to the preserved model population in Table 1 was lower (modified Jaccard Index of 0.605).

As reported earlier (Trafny 2013) and confirmed here, Pseudomonas spp. is the most common OTU inhabiting MWFs. Thus, an elucidation at species level was an additional objective of this study. Pseudomonas spp. are closely related to each other (Cornelis 2008) that makes it challenging to achieve a classification at this level by using only one house-keeping gene. Nevertheless, the data presented here clearly show that the P. oleovorans/pseudoalcaligenes group is most prominent at species level. Some strains of P. pseudoalcaligenes were recently renamed to P. oleovorans (Saha et al. 2010), which is partially reflected in the databases. Apart from representatives of this group, mainly P. stutzeri and to some extent P. aeruginosa were detectable at higher median abundances. PMA-treatment led to a shift in the detection of the different species: In 'bio-concept' fluids, the P. oleovorans/pseudoalcaligenes group became more prominent (Fig. 3a), while the relative abundance of P. stutzeri decreased. In preserved fluids, both P. stutzeri and P. mendocina remained detectable at higher abundances after the PMA treatment (Fig. 3b).

It might be reasonable to assume that microbial populations in MWFs change over time as metals leach into the fluid and MWF concentrations and pH change. Thus, any sampling represents a snapshot that may or may not reflect the true microbial state of the fluid. To get at least an idea of those presumed changes, samples from both fluid types at a random time point t from 30 systems (21 using 'bio-concept’, 9 containing preserved fluids) were taken and resampled 30 days later at exactly the same spot. Subsequently, the bacterial populations without and with PMA treatment were compared by Bray–Curtis cluster analyses. Untreated samples showed a similarity of 70% and PMA-treated samples were 65% similar (Fig. 4). The most significant changes were observed in a preserved fluid where the population shifted from mainly containing Pseudomonas spp., Mycobacterium spp. and Sphingomonas spp. to a population mainly containing Comamonas spp. and Morganella spp. (36-7% similarity). The slightest changes were observed in a 'bio-concept' fluid where shifts were only observable within OTUs already in majority (87-5% similarity). It is important to note that
any of the systems at either time point were considered ‘healthy’ and no treatment with additives occurred in between. However, PMA-treated samples were significantly less similar than untreated samples \((P = 0.01)\) as calculated by a two-way ANOVA with a significance level of 0.05 (fluid type vs pretreatment). This might suggest that fluctuations in total were rather stable but changes in viability and/or cellular stability occurred. On the other hand, there were no significant differences between ‘bio-concept’ and preserved fluids \((P = 0.17)\).

Massive parallel 16S rRNA gene tag sequencing was also applied to look for archaea. However, archaeal DNA was only present in 2 of the 78 samples analysed. All reads were classified into the genus of *Methanobrevibacter* spp. Notably, they were no longer detectable upon PMA treatment suggesting that only detectable DNA and/or compromised cells were present, rather than active microbial cells. The main species detected, *Methanobrevibacter smithii*, was described as a colonizer of the human gut system (Horz and Conrads 2010), thus they probably had their origin from human sources.

A limitation of this study is the fact that the investigation was limited to the liquid phase of the metalworking system. It is well known that micro-organisms often grow in dense, multicellular communities called biofilms that, among others, offer further protection from attack by antimicrobials. Nevertheless, a recent study (Trafny et al. 2015) demonstrated that neither the number of bacteria nor the size of the populations varied significantly between biofilms sourced from preserved and nonpreserved systems. As this particular publication was based on cultivation, further studies based on next-generation sequencing are needed to estimate the diversity in metal industry-based biofilms.

**Materials and methods**

**MWF samples**

All samples were mineral oil-based, in-use metalworking fluids either preserved or bactericide free (‘bio-concept’), collected from systems applying a wide range of
Sampling

Sampling was conducted worldwide by skilled field workers and stored/transported at ambient temperatures between 1 day and 10 days before processing. Later, samples were sourced in Switzerland only, to guarantee transportation time between sampling and processing to be below 24 h. No significant changes were observed between populations with various transportation times.

Isolation of bacterial DNA

Genomic DNA (gDNA) was isolated from MWF samples using the PowerSoil® DNA Isolation Kit from MO BIO Laboratories (Carlsbad, CA, USA). This Kit is suitable for isolating gDNA from various environmental samples (Feinstein et al. 2009; Francy et al. 2009). 750 µl of each sample was used for analysis of the OTUs at genera level (with and without PMA pretreatment). For the analysis of *Pseudomonas* spp. at species level and the time-lapse study, only the ‘Swiss’ samples were used.
MWF sample was taken as starting material, and isolation was performed as described by the manufacturer. The extracted gDNA was stored at \(-21^\circ C\).

**PMA treatment**

Bacterial cells for PMA treatment were isolated in duplicate by gradient centrifugation as described elsewhere (Di Maiuta 2010). The duplicates were pooled, centrifuged at 10,000 g for 3 min, and the pellet resuspended in 500 l PBS. PMA treatment and cross-linking was performed as described by Nocker *et al.* (2007).

**Preparation of amplicon libraries**

Amplicon libraries were generated as recommended by Roche in the 454 Life Sciences Genome Sequencer System manual and as described by others (Dethlefsen *et al.* 2008; Lauber *et al.* 2009). 16S rRNA fragment libraries were obtained by amplifying two partial 16S rRNA gene regions by means of the fusion primers. Fusion primers were generated from the bacterial universal primer pair EUB8m_f and EUB515_r to amplify 16S rRNA hypervariable regions V1, V2 and V3. 25 l of Qiagen HotStarTaq Master Mix, 2 l of each primer (10 l mol l\(^{-1}\)) and 5-10 l (approx. 20–30 ng) of the extracted DNA were adjusted with H\(_2\)O to a final volume of 50 l. Cycling parameters included an initial denaturation for 15 min at 95°C, 30 cycles of 45 s at 94°C, 45 s at 52°C and 1 min at 72°C, and a final extension for 10 min at 72°C. Two independent 50 l PCR reactions were performed for each sample and the products were pooled prior to purification with the Qiagen (Hilden, Germany) QIAquick PCR purification kit. The purified samples were sent to Microsynth AG (Balgach, Switzerland) where the samples were pooled in equimolar amounts, immobilized onto DNA capture beads, amplified by emulsion-based clonal amplification and analysed on the Roche 454 Genome Sequencer FLX system (Margulies *et al.* 2005).
Data processing and analysis

Signal processing was performed using the Roche 454 Genome Sequencer FLX system software. The GS FLX Titanium chemistry used for this study allows a read length of approx. 350 bases, according to the manufacturer’s instructions (Roche, Rotkreuz, Switzerland). Low-quality sequences were removed on the basis of sequence length, recognition of the tag sequence and on the sequence quality score. Sorted sequences were analysed using the tools provided in the pyrosequencing analysis pipeline at the Ribosomal Database Project (RDP II) Website (Cole et al. 2009). Sequences shorter than 250 bp and containing an undefined base were excluded. Firstly, to perform taxonomy-based analyses the assignment of the taxonomy was performed using the naive Bayesian rRNA classifier (Wang et al. 2007) with a bootstrap confidence threshold of 60% (Jones et al. 2009). Secondly, sequence batches from one sample sharing the same tag were aligned using the fast, secondary-structure aware ‘infernal’ aligner (Nawrocki and Eddy 2007) and complete clustering of the sequences was performed by means of the complete linkage clustering method. The cluster files were used to perform taxonmetry-dependent analyses by clustering the reads in OTUs used to calculate ecological metrics such as the coverage of the libraries. Representative sequences among all of the investigated libraries were selected by combining all sequence reads to one single alignment, followed by de-replication of the data based on cluster analysis at 3% sequence divergence.

Amplification of archaeal DNA

Several independent primer pairs were evaluated (Klindworth et al. 2013; Takahashi et al. 2014). Not knowing about the occurrence of archaeal DNA in the sample subset, polymerase chain reactions (PCR) were run with randomly selected samples in order to identify optimal PCR conditions. Using a reference DNA as template, the primer pair oMT095/096 produced a 480-bp fragment representing archaeal 16S rRNA genes.

Statistics

Statistics were calculated using Excel 2016 for Mac including the Analysis ToolPak add-in to perform the two-way ANOVA.

Conflict of Interest

This study was financed by Blaser Swisslube AG and all samples were sourced from customers of this company.

References

Anderson, D.I. and Hughes, D. (2014) Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 12, 465–478.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M. et al. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37, D141–D145.

Cornelis, P. (ed.) (2008) Pseudomonas. Genomics and Molecular Biology. Norfolk: Caister Academic Press.

Dethlefsen, L., Huse, S., Sogin, M.L. and Relman, D.A. (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 18, e280.

Di Maiuta, N. (2010) Characterisation of mixed microbial populations in white mineral dispersions. PhD Thesis, University of Warwick Library, http://wrap.warwick.ac.uk/id/eprint/3388.
Dilger, S., Fluri, A. and Sonntag, H.-G. (2005) Bacterial contamination of preserved and non-preserved metalworking fluids. Int J Hyg Environ Health 208, 467–476.

Epstein, S.S. (2013) The phenomenon of microbial uncultivability. Curr Opin Microbiol 16, 636–642.

Feinstein, L.M., Sul, W.J. and Blackwood, C.B. (2009) Assessment of bias associated with incomplete extraction of microbial DNA from soil. Appl Environ Microbiol 75, 5428–5433.

Francy, D.S., Bushon, R.N., Brady, A.M., Bertke, E.E., Kephart, C.M., Likirdopulos, C.A., Mailot, B.E., Schafer, F.W. 3rd et al. (2009) Comparison of traditional and molecular analytical methods for detecting biological agents in raw and drinking water following ultrafiltration. J Appl Microbiol 107, 1479–1491.

van der Gast, C.J., Whiteley, A.S., Lilley, A.K., Knowles, C.J. and Thompson, I.P. (2003) Bacterial community structure and function in a metal-working fluid. Environ Microbiol 5, 453–461.

Gilbert, Y., Veillette, M. and Duchaine, C. (2010) Metalworking fluid biodiversity characterization. J Appl Microbiol 108, 437–449.

Horz, H.P. and Conrads, G. (2010) The discussion goes on: what is the role of euryarchaeota in humans? Archaea 2010, 967271.

Jones, R.T., Robeson, M.S., Lauber, C.T., Hamady, M., Knight, R. and Fierer, N. (2009) A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. ISME J 3, 442–453.

Kapoor, R., Selvaraju, S.B. and Yadav, J.S. (2014) Extended tracking of the microbial community structure and dynamics in an industrial synthetic metalworking fluid system. FEMS Microbiol Ecol 87, 664–677.

Klindworth, A., Pruesse, E., Schweer, T., Quast, C., Horn, M. and Glöckner, F.O. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41, e1.

Lauber, C.L., Hamady, M., Knight, R. and Fierer, N. (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl Environ Microbiol 75, 5111–5120.

Maier, L.E., Lampel, H.P., Bhutani, T. and Jacob, S.E. (2009) Hand dermatitis: a focus on allergic contact dermatitis to biocides. Dermatol Clin 27, 251–264.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S. et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376–380.

Nawrocki, E.P. and Eddy, S.R. (2007) Query-dependent banding (QDB) for faster RNA similarity searches. PLoS Comput Biol 3, e56.

Nocker, A., Sossa-Fernandez, P., Burr, M.D. and Camper, A.K. (2007) Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol 73, 5111–5117.

Pakpour, S., Scott, J.A., Turvey, S.E., Brook, J.R., Takaro, T.K., Sears, M.R. and Klironomos, J. (2016) Presence of archaea in the indoor environment and their relationship with housing characteristics. Microb Ecol 72, 305–312.

Passman, F.J. (2006) Microbiology of metalworking fluids. In Metalworking Fluids, 2nd ed, ed. Byers, J.P. pp. 195–229. Boca Raton, FL: CRC Press.

Passman, F.J. and Küenzi, P. (2015) A differential adenosine triphosphate test method for differentiating between bacterial and fungal contamination in water-miscible metalworking fluids. Int Biodeterior Biodegradation 99, 129–137.

Rezzonico, F., Vogel, G., Duffy, B. and Tonolla, M. (2010) Application of whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification and clustering analysis of pantoea species. Appl Environ Microbiol 76, 4497–4509.

Rossmore, H.W. (1993) Biostatic fluids, friendly bacteria, and other myths in metalworking microbiology. J Soc Tribologists Lub Eng 49, 253–260.

Saha, R., Sproer, C., Beck, B. and Bagley, S. (2010) Pseudomonas oleovorans subsp. lubricantis subsp. nov., and reclassification of Pseudomonas pseudoalcaligenes ATCC 1740(T) as later synonym of Pseudomonas oleovorans ATCC 8062(T). Curr Microbiol 60, 294–300.

Simpson, A.T., Stear, M., Groves, J.A., Piney, M., Bradley, S.D., Stagg, S. and Crook, B. (2003) Occupational exposure to metalworking fluid mist and sump fluid contaminants. Ann Occup Hyg 47, 17–30.

Takahashi, S., Tomita, I., Nishiooka, K., Hisada, T. and Nishijima, M. (2014) Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. PLoS ONE 9, e105992.

Theaker, D. and Thompson, I. (2010) The industrial consequences of microbial deterioration of metal-working fluids. In Handbook of Hydrocarbon and Lipid Microbiology, ed. Timmis, K.D. pp. 2641–2650. Berlin, Heidelberg: Springer-Verlag.

Trafny, E.L. (2013) Microorganisms in metalworking fluids: current issues in research and management. Int J Occup Environ Health 26, 4–15.

Trafny, E.L., Lewandowski, R., Kozlowska, K., Zawistowska-Marciniak, I. and Stepinska, M. (2015) Microbial contamination and biofilms on machines of metal industry using metalworking fluids with or without biocides. Int Biodeterior Biodegradation 99, 31–38.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007) Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73, 5261–5267.

Yue, J.C. and Clayton, M.K. (2005) A similarity measure based on species proportions. Commun Stat Theo Methods 34, 2123–2131.