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MINI-REVIEW

The microbiology of metalworking fluids

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Abstract Metalworking fluids (MWFs) are complex mixtures of chemicals and are indispensable materials in industry. They are used as cooling and lubricating agents in different machining process such as grinding, milling, and cutting. The quality of MWFs is affected by physical, chemical, and microbial contaminates. In particular, MWFs are highly vulnerable to microbial contamination, which may act both as potential pathogens and deteriorgens. Microbial contamination is of major concern due to potential health hazards such as skin dermatitis and hypersensitivity pneumonitis. The contaminated MWFs can exhibit high degrees of microbial loading, ranging from 10^4 to 10^{10} colony-forming units (CFU)/ml. Wide varieties of microorganisms are reported to colonize MWFs. Traditional culturing techniques are not only laborious and time consuming but also underestimate the actual distribution of the microorganisms present in the contaminated MWFs. Therefore, rapid molecular methods such as real-time PCR and fluorescent in situ hybridization are implemented to monitor the microbial load. In industry, biocides are presently used to control microbial contamination. However, it has its own disadvantages and therefore, in recent years, alternative methods such as UV irradiation were evaluated to reduce microbial contamination in MWFs. Microbes inhabiting the MWF are also capable of forming biofilm which is detrimental to the MWF system. Biofilm is resistant to common disinfectant methods, and thus further research and development is required to effectively control its formation within MWF systems. This review is intended to discuss the overall microbiological aspects of MWF.

Keywords Metalworking fluids · Real-time PCR · FISH · Biocides · Biofilm

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Introduction

Metalworking fluids (MWFs) are complex mixtures of chemicals used in industries for cooling and lubrication of different types of machining processes such as turning, grinding, milling, and cutting (Chang et al. 2004; Cheng et al. 2005; Gilbert et al. 2010a; Selvaraju et al. 2011; Rossmoore 1995) (Fig. 1). Due to their ability to increase tool life by improving the finish of a work piece and preventing corrosion, MWFs are vital requisites in industrial processes (van der Gast et al. 2003; van der Gast and Thompson 2004). There are mainly four classes of MWFs: (1) straight oil, (2) soluble oil (emulsifiable oil), (3) semi-synthetic, and (4) synthetic (Gauthier 2003; NIOSH 2001; Robertson et al. 1988). Various additives such as biocides and corrosion inhibitors are often added to enhance the performance and lifespan of the MWFs (Selvaraju et al. 2005, 2011; Virji et al. 2000) (Table 1).

MWFs are highly susceptible to physical, chemical, and microbial contamination. The microbes present in MWFs are of major concern due to their capability to act as potential pathogens and/or deteriorgens (Rossmoore 1995; van der Gast et al. 2001). MWF formulations commonly consist of petroleum oil (1 to 5 %), petroleum sulfonates (0.1 to 0.5 %), and fatty acids (less than 0.1 %, mainly linoleic and oleic acids) which serve as the primary sources of carbon for

Fig. 1 An example of a machine that uses metalworking fluids

microbial growth (Foxall-VanAken et al. 1986). High levels of contamination ranging from 10^4 to 10^{10} CFU/ml have been reported in MWFs (Mattsby-Blatzer et al. 1989; Sloyer et al. 2002; van der Gast et al. 2003). A wide variety of microorganisms such as *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Alcaligenes*, sulfate-reducing bacteria (SRB), and *Acinetobacter* are known to inhabit MWFs (Mattsby-Blatzer et al. 1989; Perkins and Angenent 2010; Sandin et al. 1991; Virji et al. 2000; van der Gast et al. 2003) (Table 2), including pathogens (opportunistic) such as *Legionella* sp., *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Bakalova et al. 2007; Elsmore 2003; Lucchesi et al. 2012). Relatively recently, a new species of *Mycobacterium* (*Mycobacterium immunogenum*) was isolated from used MWFs that was associated with hypersensitivity pneumonitis (Moore et al. 2000; Wallace et al. 2002; Rhodes et al. 2011). Kampfer et al. (2009) isolated a novel Gram-positive bacterium, *Tessaracoccus lubricantis*, from contaminated MWF. Although a wide variety of microorganisms are found in MWFs, the dominant groups of microorganisms that colonize MWFs belong to the genera *Pseudomonas* and *Mycobacterium* (Baecker et al. 1989; Khan and Yadav 2004; Narayan Rao et al. 2011; Selvaraju et al. 2011; Wilson et al. 2001). The commonly found pseudomonads in MWFs are *Pseudomonas oleovorans* subsp. *oleovorans*, *Pseudomonas oleovorans* subsp. *pseudoalcaligenes*, *Pseudomonas fluorescens*, *P. aeruginosa*, and the newly described *Pseudomonas oleovorans* subsp. *lubricantis* (Gilbert et al. 2010a; Khan and Yadav 2004; Saha et al. 2010b, 2011a, b; van der Gast et al. 2003). A recent study by Murat et al. (2012) reported that metal types and the nature of MWF play a part in the microbial composition of contaminated MWF. It was found that Gram-negative rods were predominant in MWFs used in non-automotive industry, whereas Gram-positive rods were more prevalent in MWFs used in automotive industry. Certain metals such as chromium, nickel, and iron used in the non-automotive industry might be associated with the growth of Gram-negative rods. Liu et al. (2010) reported that species of *Exiguobacterium*, *Micrococcus*, and *Staphylococcus capitis* were the dominant airborne bacteria in MWF environments. Fungi (yeast and molds) are also found as contaminates in MWFs. Species of *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium*, and *Cepalosporium* are commonly isolated from contaminated MWFs. Fungi are associated with hypersensitivity pneumonitis, asthma, and other allergies. Certain species are also known to produce toxic metabolites such as mycotoxins (Liu et al. 2010; NIOSH 2001).

Biodeterioration of MWFs due to microbial contamination has several detrimental effects. It changes the stability of the emulsion by altering fluid viscosity, increasing the rate of corrosion leading to leaks in MWF systems, reducing tool life (Burge 1996; NIOSH 2001; Rossmoore 1995). Biofilms and fungal growth cause clogging of the machining systems (Hill 1978; Kinniment and Wimpenny 1990; Lucchesi et al. 2012; Mattsby-Blatzer et al. 1989; Rossmoore 1995). Apart from fluid degradation, microorganism in MWFs poses potential health hazards to workers. For example, SRB produce hydrogen sulfide (H_2S) which acts as a carcinogen affecting brain, lungs, and heart (Arnold et al. 1985; Guidotti 1996; Stear 2005). Gram-negative bacteria produce endotoxins that lead to adverse pulmonary infections (Brown et al. 2000; Gordon 2004; Laitinen et al. 1999; Passman 2008; Selvaraju et al. 2011). Further, microbial contamination of fluid mists generated during various machining process causes asthma, hypersensitivity pneumonitis, and lung cancer (Gauthier 2003; Robins et al. 1997; Gilbert et al. 2010b; Selvaraju et al. 2008a, b).

In view of the facts stated earlier, the goals of this review article are to discuss (a) microbial contamination, (b) the rapid methods of detection, (c) the methods of disinfection, and (d) biofilm in MWF systems. This is the first review article in the area of MWFs to discuss the microbiological aspects of MWFs. A number of methodologies have been employed to study and reduce the microbial load of MWFs (Saha 2009; Selvaraju et al. 2005; Rudnick 2003). Historically, traditional culturing techniques were implemented to study the microbial diversity and to determine the level of contamination in MWF, but only a small proportion (1 %) of culturable bacteria was recovered using

Table 1 Chemical composition of MWFs (adapted from NIOSH (2001))

Component	Function	Straight oils	Soluble oils	Semisynthetics	Synthetics
Water	Acts as coolant solvent, diluent	Dissolved 10–500 ppm/wt[a]	5–40 parts/part concentrate	10–40 parts/part concentrate	10–40 parts/part concentrate
Mineral oil	Carries lubrication	60–100 %	30–85 %	5–30 %	[b]
Emulsifier	Emulsifies	[b]	5–20 %	5–10 %	5–10 %
Chelating agents	Tie-up ions in solution	[b]	0–1 %	0–1 %	0–1 %
Coupling agents	Stabilize	[b]	1–3 %	1–3 %	1–3 %
Viscosity index improvers	Maintain viscosity	[c]	[b]	[b]	[b]
Detergent	Prevents deposit formation	[c]	[c]	[c]	[c]
Plasticizer	Reduces tackiness	[b]	[c]	[c]	[c]
Antimist agent	Reduces misting	[c]	[c]	[b]	[b]
Antiweld agent	Prevents welding	0–20 %	0–20 %	0–10 %	0–10 %
Oiliness agent	Increases film strength	[c]	[b]	[b]	[b]
Surfactant wetting agent	Reduces surface tension	0–10 %	5–20 %	10–20 %	10–20 %
Dispersants	Prevent fine agglomeration and deposit formation	[c]	[b]	[b]	[b]
Passivator	Prevents staining	[c]	[b]	[b]	[b]
Anti-foaming agents	Prevent foaming	0–500 ppm	0–500 ppm	0–500 ppm	0–500 ppm
Alkaline reserve	Acts as buffer control	[b]	2–5 %	2–5 %	2–5 %
Dyes	Identify, leak detection	[b]	0–500 ppm	0–500 ppm	0–500 ppm
Odorant	Masks odor	[c]	[c]	[c]	[c]
Corrosion inhibitors, anti-rust	Prevent rust film barrier	0–10 %	3–10 %	10–20 %	10–20 %
Biocides, bioresistant components	Control bacterial and fungal contaminants	[b]	0–2 %	0–2 %	0–2 %
Extreme pressure additives	Act as reaction lubricant films	0–40 %	0–20 %	0–10 %	0–10 %

[a] Dissolved water concentrations in mineral oils range from 10 to 100 mol per million carbon atoms, depending on ambient humidity and temperature

[b] Not present in this MWF class

[c] Usually present in this MWF class

bacteriological media, thus underestimating the actual microbial load in samples (Amann et al. 1995; Khan and Yadav 2004; Saha et al. 2011a; Ward et al. 1995). To overcome the problems associated with the determination of bacterial diversity and the related level of contamination in different environmental and clinical samples, molecular approaches like quantitative real-time PCR (qPCR) and fluorescent in situ hybridization (FISH) are presently used (Amann et al. 1995; Khan and Yadav 2004; Suzuki et al. 2004; Rhodes et al. 2008, 2011). Several workers have also attempted to perform dry machining and use mist suppressants to control microbial activity (Klocke and Eisenblatter 1997; Marano et al. 1997; Aoyama 2002). However, these methodologies were not very effective because dry machining cannot be used in all machining conditions and mist suppressants are susceptible to shear effects due to rise in temperature (Rudnick 2003).

Biocides

In order to disinfect and reduce the microbial content of MWFs, biocides and bioresistant chemicals such alkanolamines, formaldehyde, and non-formaldehyde-based biocides are often used. However, limited information is available on the evaluation of these biocides to determine their relative efficacy (Falkinham 2009; Selvaraju et al. 2005). This can lead to skin irritation (leading to dermatitis), corrosion, microbial resistance, and toxicity along with added handling and disposal cost (Sandin et al. 1990; Rossmoore 1995; Lin et al. 1999; Selvaraju et al. 2008a, b; Skerlos et al. 2000). In recent years, multiple studies were conducted by Selvaraju et al. (2005, 2008, 2011) to evaluate the potential effectiveness of some of the commonly used biocides such as formaldehyde releasing, isothiazolone, and phenoloc biocide in MWFs (synthetic and semi-synthetic)

Table 2 Studies of microbial communities in contaminated MWFs

Title	Reference
Microbiological contaminants of metalworking fluids in service	Baecker et al. (1989)
Bacterial microflora of contaminated metalworking fluids	Bakalova et al. (2007)
Microbiology of metalworking fluids: pilot studies of a large-scale exposure assessment experience	Burge (1996)
The survival of *Legionella pneumophilia* in dilute metalworking fluids	Elsmore (2003)
Metalworking fluids: oil mist and beyond	Gauthier (2003)
Metalworking fluids biodiversity characterization	Gilbert et al. (2010a, b)
Investigation into the nature and extent of microbial contamination present in a commercial metalworking fluid	Lin et al. (1999)
Occurrence and characterization of culturable bacteria and fungi in metalworking environments	Liu et al. (2010)
Evaluation of bacterial contamination and control methods in soluble metalworking fluids	Marchand et al. (2010)
Microbial growth and accumulation in industrial metalworking fluids	Mattsby-Blatzer et al. (1989)
Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria	Moore et al. (2000)
Factors influencing the microbial composition of metalworking fluids and potential implications for machine operator's lung	Murat et al. (2012)
Experimental investigation of microbial contamination of nano cutting fluids with Cnt inclusion	Narayan Rao et al. (2011)
Metalworking fluid microbes—what we need to know to successfully understand cause and effect relationships	Passman (2008)
Potential pathogenic bacteria in metalworking fluids and aerosols from machining facility	Perkins and Angenent (2010)
Microbiology of metalworking fluids: deterioration, disease and disposal	Rossmoore (1995)
Rapid bacterial counts in metalworking fluids	Sloyer et al. (2002)
Identification and characterization of bacterial populations of an in-use metalworking fluid by phenotypic and genotypic methodology	van der Gast et al. (2001)
Bacterial community structure and function in a metalworking fluid	van der Gast et al. (2003)
Identifying the determinants of viable microorganisms in the air and bulk metalworking fluids	Virji et al. (2000)

against the predominant bacteria such as *M. immunogenum* and *P. fluorescens*. In one of the studies, a marked increase in biocidal resistance was observed for both test organisms when present in MWF matrix (Selvaraju et al. 2005). In another study with semi-synthetic MWF, it was observed that higher amounts of formaldehyde-releasing biocides were required to completely inactivate *M. immunogenum* compared to non-formaldehyde-releasing biocides (Selvaraju et al. 2011). Based on these observations, it was recommended that, to effectively control microbial contamination using biocides, proper fluid management practices including routine monitoring of the critical factors in an industrial setting would be required.

Ultraviolet irradiation

Ultraviolet light (UV) or radiation encompasses light with wavelength shorter than the violet end of the visible spectrum. Light with wavelengths between 200 and 400 nm is considered to be UV. There are four ranges of UV: (1) UVA (315–400 nm), (2) UVB (280–315 nm), (3) UVC (200–280 nm), and (4) VUV (vacuum ultraviolet) in the range of 100–200 nm. The UVC range is the most critical from the disinfection perspective as it is known as the germicidal range and is very effective in inactivating microorganisms (Asano et al. 2007)

The UVC range is absorbed by the microbial cells causing photochemical damage to the nucleic acid. Dimerization of the nucleotides (mainly pyrimidine) takes place in both the DNA and the RNA molecules present in the cell, which prevent replication and cell division, leading to cell death. The extent of cell damage depends on the dosage of the UV irradiation and resistance of the microbe to UV. UV dose is the product of intensity of the UV and exposure time. It is expressed in millijoules per square centimeter (mJ/cm^2) or milliwatt seconds per square centimeter (mWs/cm^2) (Asano et al. 2007).

UV irradiation has successfully been implemented to reduce microbial load in water (Severein et al. 1983; Blatchley et al. 1998) and air (Riley and Nardella 1989) and for surface sterilization (Katara et al. 2008). The main advantage of UV over biocides is that it does not produce any undesirable by-products and does not require additional storage or disposal.

John and Phillips (2002) demonstrated that MWF spiked with *P. fluorescens* (10^7 CFU/ml) and irradiated with a 6-W submerged non-glass UV lamp resulted in two-log reduction in 60 mins. Peppiatt and Shama (2000) irradiated microbially contaminated commercial MWF using a thin film

contractor at a flow rate of 1.8×10^{-2} m^3s^{-1} at a UV dose of 44.5 mW s/cm^3. The MWF was irradiated in the form of "bells" generated with a specially designed nozzle which finally led to a decline of 10^6 to 10^7 CFU/ml that occurred within 6–8 h. Similar works carried out for endotoxin inactivation of drinking water by UV irradiation suggested that 1 to 50 endotoxin units (EU/ml) could effectively be inactivated with UV fluences of up to 500 mJ/cm^2 (Anderson et al. 2003).

A recent study was conducted by Saha (2009) to determine the parameters that could be utilized to design an in-line UV reactor, which could be used in enclosed machining operations. Two semi-synthetic unused 5 % diluted MWF samples were spiked with different concentrations (10^4–10^7 colony-forming units, CFU/ml) of three indicator bacteria, *P. fluorescens, P. oleovorans* subsp. *lubricantis*, and *M. chelonae*, separately as well as in mixed culture combinations. The spiked MWF samples were irradiated with a high-intensity (192 μW/cm^2, 55 W) UV lamp for different exposure times under both static and mixed conditions. After exposure, viable counts were determined from the irradiated experimental samples. Under static conditions, only 56 % reduction in viable count was observed within 10 min of exposure for *P. fluorescens* with a cell concentration of 10^7 CFU/ml. In contrast, under mixed conditions, 99 % reduction was achieved within 2 min for both *P. fluorescens* and *P. oleovorans* subsp. *lubricantis*, whereas only 74 % reduction was observed for *M. chelonae*. However, with a low concentration of cells (104 CFU/ml), 99.99 % reduction was observed for the *Pseudomonas* species and 82 % for *M. chelonae* under mixed conditions. Similar results were obtained with a mixed-culture combination of the indicator bacteria. The experimental results indicated that with a combination of high-intensity lamp and mixing, UV could be successfully used as a means of disinfection of MWF within a short exposure time for enclosed machining systems.

Rapid and accurate monitoring of microbial contaminations in MWFs requires the implementation of molecular techniques such as real-time PCR and FISH.

Real-time PCR

Conventional PCR was developed by Kary Mullis in 1980 (Valasek and Repa 2005) to exponentially amplify target sequences based on end-point detection using thermotolerant DNA polymerase enzyme. In the conventional PCR technique, it is not possible to quantify the starting DNA and monitor the progress of the reaction. Real-time PCR (qPCR) was first performed by Higuchi et al. (1993) at Roche Molecular Systems using the fluorescent dye ethidium bromide. A video camera was used to detect and monitor the fluorescence of the dye under UV light with the progress of the PCR reaction as ethidium bromide fluoresce strongly under UV when incorporated in double-stranded DNA (Higuchi et al. 1993). Presently, qPCR is employed to detect and quantify specific DNA sequences using fluorescently labeled probes. The real-time instrument detects the fluorescent signal generated at each cycle, which is directly proportional to the concentration of the DNA present in the reaction, and the cycle at which the signal detected due to amplification exceeds the background signal is known as the "threshold cycle" (C_t) (Arya et al. 2005). The C_t value is inversely proportional to the nucleic acid concentration in the starting reaction (Suzuki et al. 2005).

qPCR utilizes different fluorescent chemistry for detecting DNA amplification in real-time: (1) hydrolysis probes (TaqMan®, molecular beacons and scorpions), (2) hybridization probes, and (3) DNA binding agents (SYBR Green I, ethidium bromide). One of the most important factors in designing qPCR assays is the selection of the chemistry for the reaction that depends on the sensitivity, specificity, and funds available for the target experiment (Arya et al. 2005; Epsy et al. 2006). Each qPCR chemistry has its own unique characteristics.

Recently, qPCR assays using different chemistry have been successfully used for the detection and enumeration of bacteria from contaminated MWFs. Khan and Yadav (2004) developed and optimized SYBR Green qPCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in MWFs. Saha et al. (2010a) developed a TaqMan assay to detect *mendocina* sublineage *Pseudomonas* species in contaminated MWFs. The rapidly growing *Mycobacterium* species, mainly *M. immunogenum*, have been detected and quantified using TaqMan probe by Rhodes et al. (2008, 2011). Veillette et al. (2008) also developed and evaluated a qPCR method for the detection and enumeration of *M. immunogenum* in MWFs. All of the methods mentioned earlier demonstrate the advantages of using the PCR-based method against the classical bacterial culture-based approach. These rapid molecular methods can be successfully and routinely used in laboratories to provide early detection of the bacteria and help in the effective management of microbial contamination of MWFs. Other culture-independent molecular techniques such as denaturing gradient gel electrophoresis were also developed for the detection and enumeration of bacteria present in MWFs (van der Gast et al. 2001).

Fluorescent in situ hybridization

The concept of in situ hybridization was first developed by Pardue and Gall (1969), Buongiorno-Nardelli and Amaldi

(1969) and John et al. (1969) using radioactively labeled DNA or RNA. With the development of fluorescent dyes, DeLong et al. in 1989 detected microbial cells using fluorescent-labeled molecular probes. This technology involves the hybridization of a fluorescently labeled oligonucleotide probe to its complimentary sequence present within the cell without altering its morphology and integrity (Moter and Gobel 2000; Amann and Fuchs 2008). FISH can be directly applied to samples without cultivation of cells and could be efficiently used to identify microbes and study their morphology and spatial distribution in a wide variety of complex environments (Amann et al. 1995). It can be used as a powerful tool for molecular diagnostic and phylogenetic studies (Ishii et al. 2004; Aminov et al. 2006; Lenaerts et al. 2007; Amann and Fuchs 2008; Gescher et al. 2008).

Fluorescent in situ hybridization (Fig. 2) includes the following steps: (1) sample fixation: fixation can be performed using chemicals like formaldehyde, paraformaldehyde, glutaraldehyde, and ethanol (Waar et al. 2005). The selection of the chemical for fixation depends on the sample and nature of the experiment. For example, Gram-negative bacteria can be fixed using 3–4 % (*v*/*v*) formaldehyde or paraformaldehyde (Tang et al. 2005); (2) sample preparation: samples are smeared on special slides coated with Teflon, gelatin, or poly-L-lysine (Amann et al. 1990). The smeared samples are air-dried and dehydrated in an ethanol series. Gram-positive bacteria are additionally treated with an enzyme such as lysozyme to make the peptidoglycan layer permeable to the probe (Meir et al. 1999); (3) hybridization: hybridization is carried out in hybridization buffer containing different concentrations of formamide to control the stringency of the reaction. The specificity of the probe is controlled by hybridization conditions such as formamide concentration, salt concentration, pH, hybridization temperature, and the sequence of the probe (Polak and McGee 1990). In general, specificity is altered by altering the stringency of the hybridization reaction (i.e., changing formamide concentration) and temperature. High formamide concentration and hybridization temperature will allow hybridization of the probe to sequence with similar homology, thus increasing the specificity of the probe (Easteal et al. 1991; Hugenholtz et al. 2001); (4) washing: post-hybridization stringency is controlled by altering the salt concentration of the washing buffer to remove unbound probes (Lathe 1985); (5) visualization: epifluorescence microscope with different filter sets is used for documentation of the hybridization images. Most of the microscopes are equipped with cameras and image analysis software which are used for the identification and enumeration of microorganisms (Manz et al. 2000). Confocal laser scanning microscope is used for measuring cells with high background such as biofilms, sludge, and tissue sections (Wagner et al. 1994; Manz et al. 1995). Flow cytometry

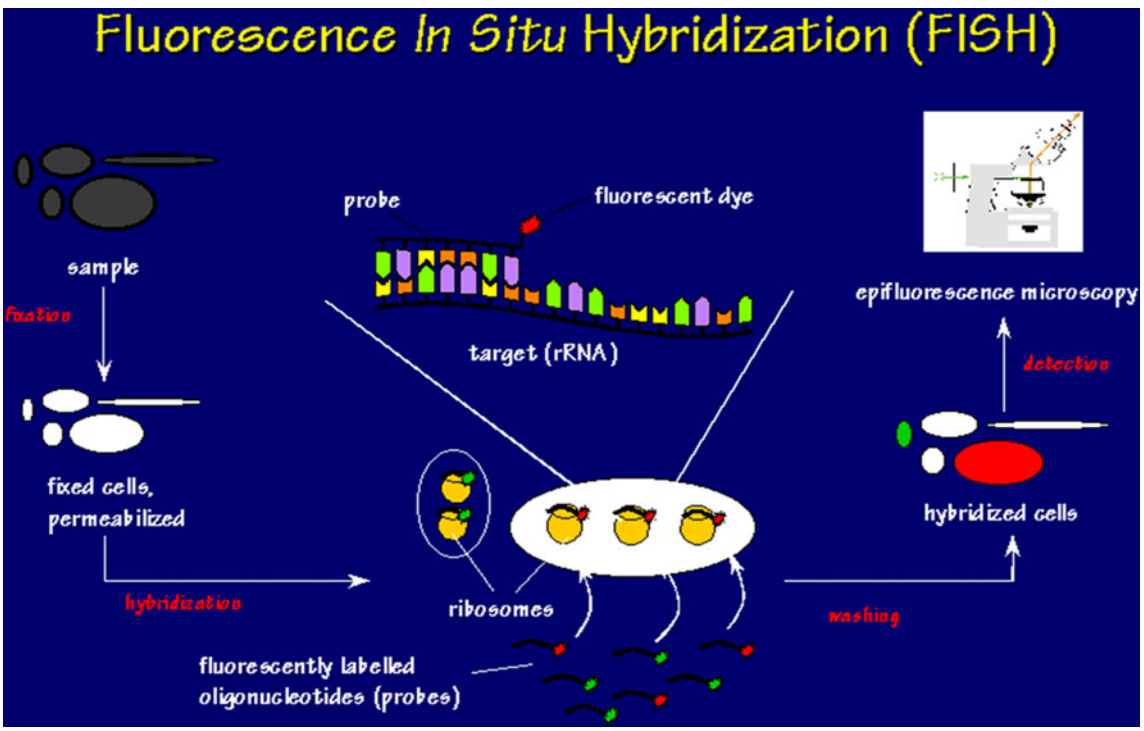

Fig. 2 Flow chart of fluorescent in situ hybridization (image courtesy: with permission from Dr. Frank Oliver Glockner)

can also be used to detect signals for quantification generated by fluorescence probes tagged to their target cells (Amann et al. 1990; Wallner et al. 1997).

FISH probes

Ribosomal RNA (16 S rRNA) molecules are commonly targeted in FISH studies for the detection and quantification of bacteria and archaea from different environments because they are ubiquitously present in high numbers in viable cells; large datasets of sequences are available, consisting of highly conserved and variable regions that are exploited to design probes (genus or species specific) depending on the objective of the investigation (Wallner et al. 1993; Amann et al. 1995). Sequence variation helps to distinguish microbes on all phylogenetic levels (Woese 1987). A wide variety of probes, for example, dsDNA, ssDNA, and synthetic oligonucleotide, can be designed and used for FISH experiments (Feldman et al. 1997). For microbial identification and quantification, small oligonucleotide probes (18–40 base pairs) are designed using an appropriate software (e.g., ARB) from the ribosomal sequence information as they are highly specific and could also easily permeabilize the microbial cell (Mahmoud et al. 2007). The probes are directly labeled with fluorescent dyes at either the 5′ or the 3′ end of the oligonucleotide. A wide variety of fluorescent dyes (fluorescin and rhodamine) can be used for labeling (Cullander 1999). The specificity of the probe depends on several factors such as sequence of the probe, size of the probe, permeability of the cells, hybridization conditions, and also labeling techniques (Rattray and Micheal 1998).

Applications of FISH

Fluorescent in situ hybridization of whole cells with oligonucleotides targeting 16S rRNA molecules has been successfully applied in different environmental and clinical samples. The microbial diversity of a wide variety of natural environments is ranging from river water system (Kenzaka et al. 1998), sea water (Tang et al. 2005), sediment (Liobet-Brossa et al. 1998), corals (Ainsworth et al. 2006), plankton assemblages (Glockner et al. 1996), snow of a high mountain lake (Weiss et al. 1996), Antarctic coastal waters (Murray et al. 1998), sulfidic and acidic mines (Kock and Schippers 2008), and the detection of raw milk spoilage organisms (Gunasekera et al. 2003). In recent years, the utilization of FISH has rapidly increased in medical diagnostics for the detection and enumeration of pathogens associated with oral infections like periodontotitis and gingivitis (Sunde et al. 2003), respiratory tract infections like cystic fibrosis (Hogardt et al. 2000), gastrointestinal disorders (Waar et al. 2005), colonic spirochaetosis (Boye et al. 1998), and implanted medical devices (Krimmer et al. 1999; Oosterhof et al. 2006).

Similarly, this technology has been implemented in MWFs for the study of culturable and non-culturable microorganisms. van der Gast et al. (2003) detected *Proteobacteria* (subclasses *alpha*, *beta*, *gamma*), *Cytophaga–Flavobacterium*, and high G+C-content gram-positive bacteria from used MWF samples collected from three different continents. Recently, Selvaraju et al. (2008a, b) developed a DNA-FISH assay for the detection of bacteria belonging to the genus *Pseudomonas* in MWFs. The levels of pseudomonads detected in different MWF samples were in the range of 1.80×10^5 to 1.05×10^6 cells/ml. Previously, Sloyer et al. (2002) developed a rapid (10 s) automated FISH method to estimate viable bacteria in MWFs and was compared against the dip–slide method presently used in the industry. The BactiFluor method was performed successfully in all the 107 MWF samples used along with 30 other metal-processing fluids. Saha et al. (2011b) also developed a FISH method for the rapid detection of rRNA group I pseudomonads in contaminated MWFs and to study the biofilm-forming capabilities of some of the predominant *Pseudomonas* species. The unique Pseudo120 probe successfully detected and enumerated the abundance and distribution of *Pseudomonas*, indicating levels between 3.2 ($\pm$ 1.1)$\times10^6$ and 5.0 ($\pm$ 2.3)$\times10^6$ cells/ml of used MWFs.

Advantages and limitations of FISH

There are several advantages of using FISH for the study of microbial communities from diverse environments. It can be directly applied to samples for the visualization of morphology and spatial distribution of both culturable and non-culturable microbes within their natural habitat (Amann et al. 1995; Hicks et al. 1992; Wagner et al. 2003). In comparison to traditional culturing techniques and PCR-based methods, it is less time consuming and more cost effective, respectively (Moter and Gobel 2000). However, it has certain limitations such as autofluorescence (Brown and Lowbury 1996), photobleaching (Moter and Gobel 2000), insufficient permeability (Krimmer et al. 1999), and low rRNA content (DeLong et al. 1989) due to the physiological state of the cells.

Biofilm

A microbial biofilm is a complex community of microorganisms growing in a biotic or abiotic surface in an aqueous environment (Donlan 2001; Lynch and Robertson 2008). It is a naturally occurring process in the environment and is a

strategy used by microorganisms to protect the cells to survive under extreme nutritional conditions (Lucchesi et al. 2012). In nature, only 10 % of bacterial cells are found in planktonic form (Lucchesi et al. 2012). Biofilm can be composed of multiple species of organisms including Gram-positive or Gram-negative bacteria along with yeast and protozoa (Bell 2001). The development of a biofilm depends on the availability of nutrients and surface for attachment (Costerton et al. 1999; Stoodley et al. 2002). Microorganisms constituting biofilm communities are less susceptible to antimicrobial agents and are thus difficult to control (Xu et al. 2000; Stewart and Costerton 2001). Some of the biofilm-related issues in the MWF industry are pipe blockage, product contamination, deterioration of industrial equipments, and biocorrosion (Lucchesi et al. 2012). Although biofilms in MWF systems have not been well studied, there are a few works documenting the role of biofilms related to MWF systems (Cook and Gaylarde 1988; Passman et al. 2000), in MWF sumps (Moore et al. 2000), flow-through reactor systems (Mattsby-Blatzer et al. 1989), disinfection treatments (Passman 2008), and biocide resistance in *P. aeruginosa* recovered from contaminated MWF (Sondossi et al. 1984). These studies indicated that cleaning and use of biocide had less impact on bacterial consortia present within the biofilms compared to planktonic bacteria present in contaminated MWFs. Due to the continuous release of microbial biomass from biofilm sources, there exists a steady concentration of microorganisms (10^7 CFU/ml) capable of growth (within 12 h after cleaning; Veillette et al. 2004), which makes control application critical in MWF systems with biofilms (Skerlos et al. 2001). A recent study by Saha et al. (2011b) demonstrated that, under a low concentration of cells, biofilm formation (Fig. 3) by some of the predominant species of *Pseudomonas* could be delayed by 24 h, indicating that implementation of suitable disinfection methods along with a proper fluid management system could prolong the life of in-use MWFs. Also, a study by Lucchesi et al. (2012) reported that the type of material used in MWF industries had little influence on biofilm development and on the

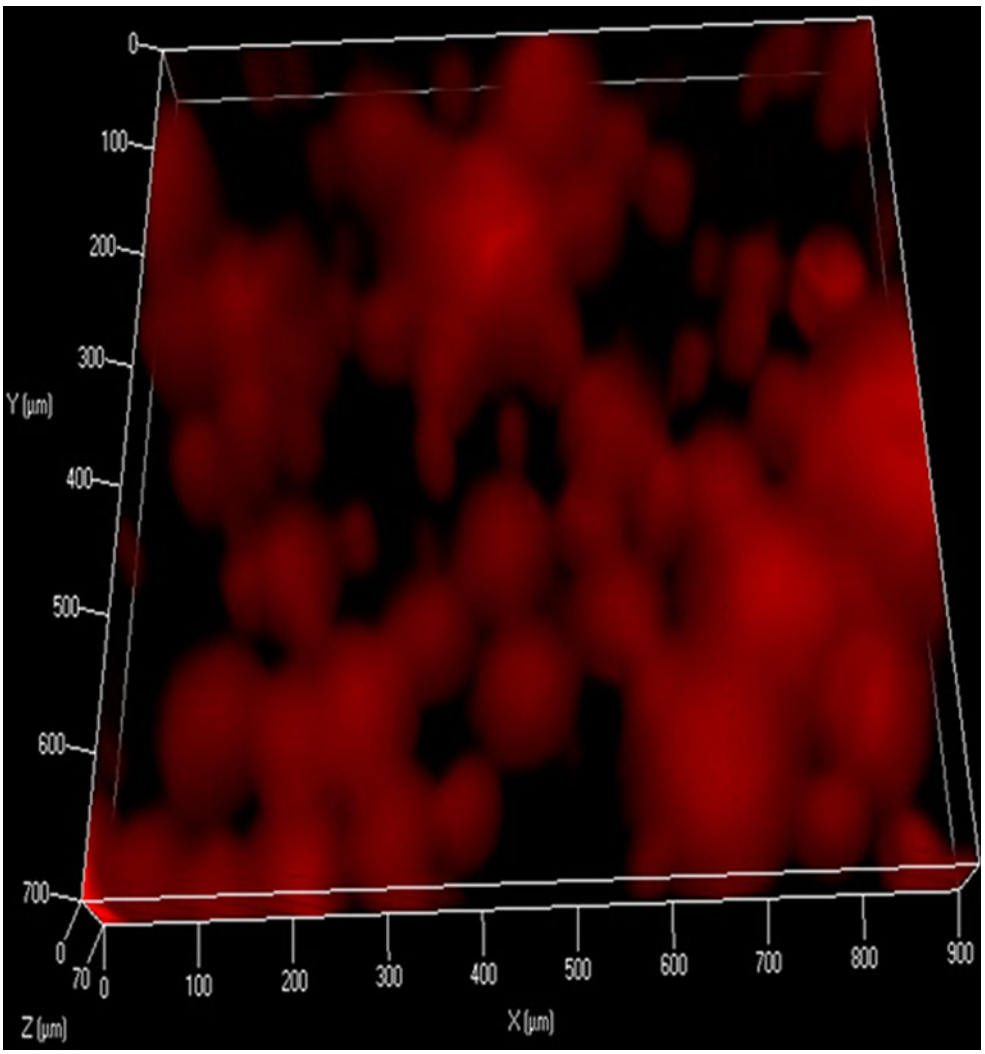

Fig. 3 Biofilm formation by *Pseudomonas oleovorans* subsp. *lubricantis* after 96 h under static condition. The biofilm is stained with the probe Pseudo 120 tagged with Cy3 fluorescent dye

concentration of biocides required to control biofilm formation. Based on these investigations, further research and development are recommended to be conducted to study the efficacy of different disinfectants on the biofilms formed in the sump of MWF systems.

Conclusion

The understanding of the diversity, composition, and biofilm-forming capabilities of the microbial community in various contaminated MWFs along with the implementation of molecular approaches will help to develop better MWF management strategies that will not only prolong the life of MWFs but will also protect the health of the workers. It will also help in the development of alternative control methods and also allow the formulation of new MWFs that are resistant to microbial colonization.

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