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MICROORGANISMS IN METALWORKING FLUIDS: CURRENT ISSUES IN RESEARCH AND MANAGEMENT

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Abstract
The microbial contamination of water miscible metalworking fluids (MWFs) is a serious problem in metal industry. A good maintenance of MWF re-circulation systems can extend the lifetime of coolants and ensure the quality of the tools produced. In MWFs, as in the other water-based environments, microorganisms usually live in the form of biofilms, the communities of bacteria and fungi attached to the surface of sumps, metal parts and also to each other. Biofilms exhibit very high resistance to biocides. The effect of biocides that are used as additives to MWFs to control the growth of the bacterial and fungal microbiomes (microorganisms characteristic to the individual coolant system) have become the subject of research only in recent years. There are also only sparse reports on the impact of biocides on microorganisms growing in biofilms in MWF installations. Fast growing mycobacteria are important members of these biofilm communities. Their presence has recently been linked with the occurrence of cases of hypersensitivity pneumonitis, a serious respiratory disorder in the metal industry employees. The new, relatively fast and inexpensive techniques to assess the species diversity within MWF microbiomes and their population size should be developed in order to control the microorganisms' proliferation in MWFs and to diminish the occupational exposure to harmful bioaerosols in metal industry.

Key words:
Metalworking fluids, Biofilm, Nontuberculous mycobacteria, Biocides

INTRODUCTION

In order to estimate the actual exposure of workers to microbial contamination of MWFs one should consider an important biological phenomenon – the growth of microorganisms in circulating coolant systems in the form of biofilms, the aggregates of microorganisms adhering to surfaces. Growth of microorganisms in biofilms significantly changes the way of assessment of the contamination level of MWFs, and guides the selection of the most effective ways to prevent fluid overgrowth in industries devoted to metal shaping. The importance of biofilm in-depth research on reducing the exposure of workers to bioaerosols in the metal industry has recently been clearly highlighted by Saha and Donorfio [1].

Biofilms are common in the installations of pharmaceutical, food and metal industries. However, the biology of biofilms in industrial settings, where multi-species biofilms occur as a rule, remains poorly characterized. The main obstacle is the lack of consensus among researchers on the analytical techniques to grow multispecies biofilms in the laboratory and to determine their susceptibility to biocides. Free-floating microbial cells both in liquid laboratory media, as well as in MWFs can be many times

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more susceptible to biocides when compared to bacteria and fungi inhabiting the biofilms in these settings. There is no possibility of eradication (complete elimination) of biofilms and this leads to a phenomenon (often observed in practice) of rapid re-growth of microorganisms population after cleaning and disinfection of MWF tanks and circulation systems [2,3].

In the European Union, the regulations regarding the permissible level of microbial contamination in MWFs have not been yet established. Also, a consensus on research techniques and the rational principles of microbiological monitoring of MWF's installations has not yet been reached [1,4]. The scarce data, accessible in the literature are difficult to interpret because different analytical procedures and sampling techniques of MWFs are used in the individual EU countries. Biocides currently used in Europe have mostly been tested for antimicrobial activity against free-floating organisms in laboratory tubes instead of bacteria and fungi living in a biofilm community. Most formulations of biocides were not verified against nontuberculous mycobacteria (NTM), especially those growing in biofilm. In the literature available to date, there is only one publication (in 2009) about the impact of three selected biocides on the biofilm of *Mycobacterium immunogenum*, a representative of NTM capable of surviving in MWFs [5]. This review aims to partially complement the microbiological data needed for risk assessment of microorganisms inhabiting the MWF ecosystem.

Occupational exposure to microorganisms

An occupational exposure to MWF microorganisms in metal industry occurs mainly by direct contact with skin or by inhalation of the oil mist [6]. In the United States more than a million workers have a daily contact with the coolant during their regular operations [7]. The most common complications in the respiratory system are asthma, upper respiratory tract infections, chronic bronchitis and hypersensitivity pneumonitis, which in its acute form can manifest the flu-like symptoms [8]. As a result of contact with MWFs some dermatological disorders may also occur, such as contact dermatitis, skin eruptions, acne, bacterial and fungal infections.

There are many potential sources of the microbial contamination of MWFs. Microorganisms may be introduced into the coolant with water used as a diluent. The microorganisms that have survived washing procedures and disinfection of tanks in the form of biofilms can resettle and multiply in distribution systems. Microbes also penetrate into the cooling system on metal parts that are used in the processing. The bioaerosol can be other source of the microbial contamination, although the predominance of Gram-negative rods in MWF microbiomes (microorganisms characteristic to the individual coolant system) suggests that contaminants may rather originate from water. The water creates optimal conditions for growth of different organisms within biofilms in the circulation systems and may occasionally contaminate the bulk water used as a diluent of the coolant [9].

The finding of NTM in coolants has recently induced great interest of researchers and physicians who work in the area of microbiology and toxicology of MWFs. The environmental *M. immunogenum* and other fast growing mycobacteria are now considered as potential etiological agents of hypersensitivity pneumonitis in workers employed in machining operations. Up to date, a total of 27 outbreaks of respiratory illnesses attributed to MWF exposure have been reported in scientific literature. However, the scrutiny search for their causative agents has not yet been successful and the knowledge regarding etiology of the respiratory disease remains limited [10].

Biocides are used to control the growth of microorganisms in MWFs. The use of biocides causes only temporary reduction in microbial population [11]. Therefore, further studies that would demonstrate a clear benefit from the use of biocides are urgently needed. The main task of

these studies is balancing the harmful effects of biocides to health of workers, e.g., inhalation exposure to potentially toxic products and allergenic effects of these substances against the profits from antimicrobial activity of biocides. The contaminated MWFs lose their properties and cause the sub-standard quality of the products manufactured in the mechanical engineering industry. Microorganisms can degrade the components of MWFs [12]. The products of acid bacteria fermentation reduce the pH of the liquid; thus, increase the risk of pitting corrosion. The formation of biofilms may interfere with liquid flow in circulatory systems by clogging them. As a result of contamination, the coolant has to be removed prematurely, and circulation systems subjected to time-consuming cleaning and disinfection procedures.

Microbiomes in metalworking fluids

The microorganisms inhabiting MWFs include both aerobic and anaerobic bacteria, which can multiply to large numbers during periods of downtime or during overnight storage when the coolant is not aerated. The most commonly isolated bacteria are aerobic Gram-negative bacteria, especially *Pseudomonas* rods, many species of Gram-positive bacteria (including *Micrococcus*, *Staphylococcus*, *Streptococcus* and *Bacillus*) and numerous species of fungi. Among the fungi are both molds and yeasts.

To assess the harmful effect of microorganisms present within MWFs one has to estimate how large is their population in the coolant of particular lathes and other machines in operation. The use of only classical microbiological methods, i.e., growing the organisms on nutrient-rich media sometimes do not allow to properly estimate their actual number, because viable but nonculturable (VBNC) or dead bacteria or fungi are not, therefore, enumerated in cutting fluids. Recent studies using molecular techniques have shown that when the bacterial concentration is relatively low, below 10^5 CFU (colony-forming units)/ml, the cultural methods underestimate by up to five orders of magnitude the actual number of bacteria in MWFs. When the density of microorganisms was above this value, both approaches, either quantitative PCR (polymerase chain reaction) or plating provided similar results (4.51×10^9 16S rRNA copies/ml with a median value of 9.23×10^7 copies/ml versus 2.36×10^9 CFU/ml with a median value of 3.05×10^7 CFU/ml, N = 44) [13]. In studies by Perkins and Angenent [14] detection of microorganisms in MWFs was carried out with direct analysis of genes encoding 16S rRNA. It has been found that a similar number of microorganisms (an average of 5.1×10^8 bacteria/ml), including culturable and nonculturable bacteria, is present in MWFs for a long periods of time (fluids were sampled in winter and summer of one year). The occurrence of potential pathogenic microorganisms, such as clinically significant *Pseudomonas* spp. and *Acinetobacter* spp. has also been shown, with the dominant species identified as *Alcaligenes faecalis*.

Many authors emphasize the relatively low diversity of bacterial and fungal species inhabiting the coolants. Using molecular techniques, such as FAME (fatty acid methyl ester analysis), DGGE (denaturing gradient gel electrophoresis) and FISH (fluorescent in situ hybridization), 179 bacterial isolates that belonged to only 11 genera and 15 species have been identified in samples of cutting fluids collected at various locations in France [15]. Analysis of 44 samples of MWF collected from 25 machine shops of different industries in Canada with culturing and molecular methods (DGGE) has revealed that maximum five different species were isolated from each sample of MWF. The dominant bacterial species were rods belonging to the family *Pseudomonadaceae*, followed by other Gram-negative bacteria: *Shewanella putrefaciens*, *Stenotrophomonas maltophilia*, *Comamonas testosteroni*, *Morganella morganii*, and *Citrobacter freundii*. Gram-positive bacteria, such as *Ochrobactrum* spp., *Brevundimonas diminuta*, and *Bacillus* spp. were also detected. Among fungi, the

representatives of the following genera: *Fusarium*, *Exophiala*, *Trichoderma*, and *Penicillium* were isolated [13].

However, there are still relatively few quantitative and qualitative studies performed on the microbiomes of MWFs with the use of molecular techniques. Most of the reports on both number and species of microorganisms in liquid cooling lubricants have originated from the studies where culturable method was employed to enumerate bacteria or fungi. Hence, it has to be borne in mind that the data cited below may underestimate the actual number of microorganisms, and may also fail to appreciate species richness within the microbiomes of these environments.

The less often coolant is exchanged (in the absence of biocides), the higher count of microorganisms has been found in MWFs. In one study of the European mineral oil during one month of its operation, the aerobic bacterial count was 3.2×10^3 CFU/ml, whereas the number of oxidase-positive Gram-negative rods was equal to 2.1×10^3 CFU/ml. After four months of the coolant's operation, the number of aerobic bacteria was 4.3×10^4 CFU/ml, and the number of oxidase-positive Gram-negative bacilli increased to 3.1×10^4 CFU/ml. The number of anaerobic bacteria in these samples was estimated to be about 2.5×10^2 CFU/ml [16].

Therefore, within three months of operation of the coolant, the bacterial count increased more than tenfold, but the balance between nonfermentative Gram-negative bacteria and a total number of bacteria did not change. The similar increase in the number of both culturable (in the nutrient-poor media) and unculturable bacteria in the coolant has been described by Veillette et al. [2]. In the first 12 hours after filling of the tank with the freshly prepared cutting fluid, the bacterial count increased to 1.6×10^3 CFU/ml. After one month of the operation, the number of culturable bacteria increased to 3.1×10^3 CFU/ml, after three months – to 2.7×10^5 CFU/ml, and subsequently, six months later reached 3.1×10^5 CFU/ml. The total number of bacteria was determined by epifluorescence microscopy and was always higher by three orders of magnitude than the number of culturable bacteria. In these samples, Gram-positive bacteria (of the genus *Micrococcus* and *Bacillus*) and *M. immunogenum* were the dominant species.

The recent study performed in Poland [17] focused on the samples of MWFs collected from three different plants, where the number of bacteria was in the range of 10 to 3.2×10^7 CFU/ml, of which from 60 to 100% was Gram-negative bacteria (a potential source of endotoxin). The dominant species was *Shewanella putrefaciens*. Among the most common species of fungi were molds of the genus *Acremonium butryi*. In these studies the number of viable but nonculturable bacteria has not been estimated.

In other recent research, the microbiological analysis of 180 samples of MWF collected in the plant engineering industry in eastern France has shown that two types of microbiomes, which are mutually exclusive, emerge in metalworking fluids. In the facilities related to the automotive industry, where cases of hypersensitivity pneumonia have been recorded, mainly Gram-positive bacteria were identified in MWFs. In 38% of these samples *M. immunogenum* were detected. Gram-negative bacteria predominantly populated the samples of MWFs from the remaining plants. Moreover, *M. immunogenum* were only sporadically detected in these coolants [18].

Mycobacterium immunogenum and other fast growing mycobacteria

Fast growing NTM are ubiquitous in the environment, especially in water bodies. They may be found in potable water and also be isolated from sterile isotonic solutions or endoscopes that were subjected to the processes of high-level disinfection. More than 90 species of these microorganisms are currently known, of which about one-third are opportunists that may cause infection in humans [19]. Among these species, *M. immunogenum*, *M. chelonae*, *M. abscessus* and *M. diernhoferi* have already been isolated from MWFs [20,21]. Mycobacteria are the etiologic

agents of atypical pneumonia as well as abscesses, septic arthritis, and osteomyelitis (bone infection). Risk factors of these infections include generalized immune disorders (including cancer, immunosuppression, and alcoholism) and pre-existing lung disease such as chronic obstructive pulmonary disease [22]. NTM are of relatively low pathogenicity. They exhibit a substantial resistance to standard tuberculostatic drugs.

The first report that an unknown species of fast growing NTM was isolated from MWF was published in 2000 [23]. These bacteria originated from the industrial plant in the United States, where an increased number of cases of hypersensitivity pneumonitis in workers employed in production of automotive parts had been observed. Intensive search for causative factor have led to the isolation of mycobacteria, which occurred in a significant number (up to 10^7 CFU/ml) in coolant tanks of machine shops. In 2001, these mycobacteria were identified as *M. immunogenum*, a new species of fast growing NTM [24]. Based on restriction patterns of PCR product obtained from amplification of the *hsp65* gene it has been shown that this species belongs to the group of fast growing NTM: *Mycobacterium abscessus* / *Mycobacterium chelonae*.

The mechanisms of the immunologically mediated pulmonary disease that may be putatively associated with the presence of *M. immunogenum* have not yet been resolved. In the experiments performed with a mouse model it has been demonstrated that there is some genetic predisposition of animals that increases the likelihood of pulmonary changes characteristic of hypersensitivity pneumonitis due to exposure to live *M. immunogenum*. However, comparable changes in the lungs of mice have also been observed after their exposure to aerosols containing killed mycobacteria or even their lysates [25]. Extensive research of immunoproteome of this organism allowed determination of 33 proteins, including those that elicited an extensive immune response in humans. Among these proteins, there are six bacterial cell wall proteins, and the other four that are secreted extracellularly [26]. These proteins may serve as potentially useful antigens in the assays that might be used to examine the level of exposure to the pathogen. The study of immunological response of the employees exposed to *M. immunogenum* revealed high levels of antibodies in their sera against this species of mycobacterium. This finding may indicate that *M. immunogenum*-contaminated MWFs are responsible for hypersensitivity pneumonitis among the exposed workers [27].

The analysis of multiple samples of MWF collected from several machine shops showed variability in the culture of mycobacteria, i.e. both positive and negative culture results have consecutively been obtained. Now, it is well known that this is a typical feature of the population of bacteria living in biofilms and is associated with the periodic spread of daughter cells from the mature biofilm [23].

In order to expedite the diagnostic process of identifying NTM in the coolant it has been proposed to use fluorescent dyes followed by microscopy to detect and enumerate mycobacteria. This method enables the detection of live mycobacteria and reduces the threshold of detection of these microorganisms by three orders of magnitude compared to the culturing methods [28]. Molecular methods, such as real-time PCR, and a simple, recently developed species-specific colorimetric-PCR assay have been used to detect and identify two species of NTM: *M. immunogenum* and *Mycobacterium chelonae* in the samples of MFWs [29]. The sensitivity of the latter method has been reported to be high, and up to 10 mycobacterial cells in pristine MWF, and 100 in the field MWF samples, respectively, could be detected with the assay. In a recent survey of 363 MWF samples originating from the machining industry plants in the United States and Europe, when RT-PCR assay was used instead of cultivation alone, *M. immunogenum* has been detected in 12.2% of U.S. samples and 39.1% of samples from Europe at concentrations exceeding 10^6 CFU/ml [30].

NTM exhibit a high resistance to disinfectants. In a study conducted recently with tap water in Italy, the presence of fast growing mycobacteria has been revealed in 62% of water samples (N = 42) [31]. From the standpoint of public health, the presence of mycobacteria in water intended for human consumption is associated with potential exposure of children, the elderly and/or individuals that are more susceptible to infection with the pathogen. In studies conducted by many research centers in the United States it has already been confirmed that NMT found in drinking water and soil may be sources of infection in humans [32]. A highly significant association has been shown for the presence of mycobacteria in MWFs and the use of hexahydrotriazine biocides to protect them from the microorganisms' overgrowth [33].

Mycobacteria exhibit the ability to grow as biofilms on animate and inanimate surfaces. This feature determines their enormous tolerance for virtually all biocidal agents available to date, even if they are used in high concentrations. Adhesion to the surface of sumps and other circulation systems of water or other liquids in which water is a basic component (e.g. coolants) prevents the elimination of these microorganisms from the system along with the flow of liquid. The prevailing view is currently that the addition of biocides to the water and coolants results in eradication of susceptible microorganisms and enables effective, albeit relatively slow proliferation of mycobacteria in these environments [34].

In recent years, the results supporting an opposite view that mycobacteria are not the principal agent causing hypersensitivity pneumonitis have been published. The authors underlined that many other bacterial and fungal taxa commonly isolated from coolants may also induce this allergenic disease [35]. The need for more scrutinized studies concerning MWF microbial ecology, as well as the prevalence of bacterial and fungal taxa in diverse machine shops is currently emphasized. These studies would help to elucidate the validity of the hypothesis on *M. immunogenum* being the microbial agent responsible for hypersensitivity pneumonitis among metal workers [36].

Biofilm

Biofilm is a consortium of microorganisms belonging to one or more species that adhere to the surface and/or to each other in an irreversible way. These microorganisms are embedded in an extracellular matrix – EPS (extracellular polymeric substance). Biofilm formation takes place in several consecutive stages – through the adhesion, formation and maturation of microcolonies, followed by development of the characteristic three-dimensional structure of biofilms. Biofilms can be formed by microorganisms on any surface, i.e. the surface of the metal filings in liquid cutting oils or in the sumps. An important feature associated with the process of adhesion is the induction of changes in metabolic processes in the adherent organisms. These changes occur through repression or activation of several genes and manifest as new phenotypic features of microbial cells. The process of biofilm formation is quite fast; even in human tissues where several immune mechanisms exist to efficiently eradicate bacteria, the mature biofilm can develop within eight hours (e.g. in burn wound) [37]. The formation of biofilm in metalworking fluids is so far unknown process; therefore, the duration of the process of biofilm formation in these environments still needs to be elucidated.

The most complex structures of natural biofilms may also include protozoa and algae. Organisms of different species, genera and even higher taxonomic categories coexisting in the biofilm participate in a complex network of reciprocal relations. The knowledge of this network is still very poor [38]. Within the network, microorganisms have the ability to communicate with each other through chemical signals, e.g. signaling molecules of quorum sensing systems. Quorum sensing systems are based on the phenomenon that any single bacterium can sense

the population density in a given ecological niche. After reaching a quorum – the appropriate number of microbial cells in a given setting, a coordinated response of all cells in the population takes place through repression or activation of specific genes. Communication between the microbial cells using low molecular weight molecules occurs within a single species or genus; however, it is also likely that communication ensues between species of Gram-negative and Gram-positive bacteria, the bacteria and yeast or molds, as well as amongst the cells of prokaryotes and eukaryotes to coordinate their behavior [39].

For the representatives of public health institutions, including those responsible for occupational medicine, the most important feature of biofilms is their high resistance to antimicrobial agents, such as antibiotics, disinfectants and other biocides. The molecular mechanisms that determine the high resistance of biofilms to antimicrobials are currently under intensive research. One of them implicates the existence of mechanical barriers, such as EPS preventing the transfer of cidal substances through the biofilm. Another putative mechanism is based on the observation that a significant proportion (> 99%) of microbial cells in biofilms are susceptible to antimicrobials. It means that less than 1% of the total microbial cells in the biofilm are completely resistant to all biocides. These resistant cells have been called persisters. They occur in biofilms of both bacteria and fungi. Their characteristic phenotype is currently the subject of extensive research.

The mechanism of resistance, which involves participation of persister cells, describes correctly the real phenomenon when biocides are used to control biofilm. In these circumstances, a significant decrease in the number of microorganisms is usually observed. However, this is only a temporary phenomenon since upon removal of the cidal agent the biofilm population returns to their initial size just after several hours or several days (depending on the speed of microbial cell division). Persisters seem to be the source of the reviving biofilms.

Microorganisms living in MWFs are capable to form biofilms [40]. The number of the publications on biofilms growing in the MWFs environment is limited. In a recent work [41], the biofilms formed by *Pseudomonas* isolates were grown on microscope slides and FISH technique was used to evaluate which species of the bacteria was actually forming the biofilm. This technique allowed the detection of live bacteria in the biofilms, both grown in the laboratory, as well as these existing in the coolant samples collected from industrial plants. The studies by Geier et al [42] have provided interesting results showing that *Mycobacterium avium*, another representative of NTM group, is capable to form biofilm under low-nutrient environmental conditions, and that oxidative stress triggered by autoinducer-2, a molecule of a quorum sensing (a bacterial communication system) stimulate biofilm development by these mycobacteria.

A relatively constant bacteria population size, approximately $>10^8$ CFU/ml, is maintained within the MWF biofilms. These are usually Gram-negative rods, frequently of the genus *Pseudomonas*. After some period of time, subsequently to the exchange of coolant in the tank, other species of Gram-negative bacteria, including *Enterobacteriaceae*, join *Pseudomonas* spp. in creating multi-species biofilms. The MWF biofilm can sometimes encompass molds and yeasts. The number of bacteria within surface-attached biofilms exceeds more than twenty times the number of planktonic (free-floating) cells in the coolant [11]. This finding suggests that even when the number of microorganisms in liquid coolant is relatively small, the real number of bacteria and fungi may be underestimated when they persist within biofilms.

Control of the microbial load in MWFs

There are two widely accepted methods to reduce the overall bacterial concentration in MWFs: cleaning and use of biocides for fluid management. These two approaches have recently been compared in one plant in France,

where four similar lathes have been subjected to four different treatments [7]. Two of the lathes were cleaned, one was cleaned in a simply way (the spoiled fluid was removed and the tool's reservoir was fed with a new one), another was subjected to major cleaning (with use of the fluid manufacturer's protocol and a special cleaner for biofilm removal). The other two were also cleansed, but once a week a biocide or even two were added to MWFs for a period of six months. The coolant samples were collected and the number of cultivable bacteria as well as their biodiversity was investigated. Unfortunately, any treatment was ineffective in removing the bacteria from MWF, although both biocides (the manufacturer's biocide: 5-choro-2-methyl-4-isothiazolin-3-one (2.63%) with 2-methyl-4-isothiazolin-3-one (0.18%), and Grotan: 1,3,5-Tris-(2-hexahyethyl) hexahydro-1,3,5-triazine) allowed controlling the number of bacterial cells and reduced the species diversity within the fluids. *Pseudomonas* sp. was dominant in all lathes examined and was not susceptible to the biocides used in that study.

The consensus regarding the level of microbial contamination of cutting oils at which the use of biocides is obligatory has not yet been reached. The British guideline for industrial plants assumes that the level of contamination above 10^6 CFU/ml requires the employment of risk assessment and afterward possibly application of a biocide [http://www.hse.gov.uk]. Frequently, the decision on the biocide application in a given industrial plant is undertaken due to the appearance of clear signs of the metabolic activity of microorganisms in liquids, such as the presence of hydrogen sulfide or ammonia, a noticeable presence of biofilms, manifested by the presence of deposits (slime) on the surface of coolants, or evident acidity of the liquid [43]. Selection of an appropriate biocide depends on the presence of other chemical compounds in liquids, such as anti-corrosion formulations and, above all, on their antimicrobial activity at high inoculum density. Periodical microbiological examination of MWF together with mask and gloves to be worn by workers has been recommended in order to minimize their exposure to the microorganisms [44]. However, it must be remembered that routine microbiological examination relates only to the floating microorganisms, while the number of bacteria and fungi in biofilms can many times exceed the counts reported in the fluids and usually this value is missed.

There are only few articles describing the efficacy of biocides against microorganisms, including mycobacteria, growing in MWFs. It has been shown that *M. immunogenum* is far more resistant (2 to 1600-fold) to the formaldehyde-releasing biocides (Grotan and Bioban), isothiazolone (Kathon), and phenolic biocide (Preventol) than *Pseudomonas fluorescent*, a Gram-negative rod commonly found in coolants. The growth of mycobacteria within MWFs increases resistance towards the biocides when compared to the organisms grown in saline. The presence of both mycobacteria and pseudomonads in mixed suspension enhances tolerance of *M. immunogenum* to both formaldehyde-releasing and non-formaldehyde biocides [45,46]. Multiple genotypes of *M. immunogenum* isolated from distinct MWFs showed different susceptibility to formaldehyde-releasing biocide Grotan and the izothiazole biocide Kathon, and these isolates were more tolerant to biocides than a reference, laboratory strain [47]. The differences in the hydrophobic and waxy cell wall of the organisms may account for this phenomenon.

In contrast to the above observations, a low dosage (0.05%) of triazine-based, methyloxazolidine-based, and formaldehyde-releasing biocidal formulations has been reported to be effective against *Mycobacterium chelonae* and *M. immunogenum* when grown in planktonic culture, even in the presence of the other bacterial and fungal species [48]. The diminished number of the channel-forming MspA-like porins in outer membrane of mycobacterial cells may be responsible for the increased

resistance (even eight fold in the porin triple mutant of *Mycobacterium smegmatis*) to biocides and enhance their survival in the hostile environment [49]. The biocide resistance of biofilm-grown *M. immunogenum* has appeared to be 3-to 100-fold higher than that of the planktonic mycobacteria [5]. Biofilms of *M. immunogenum* examined in this study were grown to the density above 10^6 cells/cm^2 on the surface of 4-mm-diameter glass, copper or galvanized steel beads for up to four weeks and challenged with a series of biocidal formulations. The results showed that the mycobacteria living in biofilms were less susceptible to biocides than *Pseudomonas alcaligenes* used as control strain in these experiments. Another study on the susceptibilities of the MWFs biofilms to biocides have demonstrated that the continuous feeding of the coolants with a trazine-based biocide at a concentration of 450–750 ppm does not reduce the number of bacteria in the biofilms, which were maintained at the density of 4.5×10^8 CFU/cm^2. Application of 1000–2500 ppm of the biocide, according to the shock strategy, significantly reduced the population of microorganisms in the biofilm to the level of 100 CFU/cm^2; however, this approach does not prevent from formation of the biofilm in the coolant environment [50].

CONCLUSIONS

More comprehensive studies of biofilms formed in MWF systems will allow development of more effective methods for monitoring microbial contamination of MWFs, assessing the effectiveness of measures to prevent contamination and thus reduce the risk of metal industry workers' exposure to harmful bio-aerosols. Detailed procedures should be developed for the exchange of the old MWFs to prevent the contamination of the new-added MWF. The relatively fast and inexpensive methods are needed to efficiently quantify and report on cell population within biofilms as well as their species composition.

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