EVALUATION OF MICROBIAL CONTAMINATION IN METALWORKING FLUIDS IN BRAZILIAN METALLURGICAL INDUSTRY

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ABSTRACT: Metalworking Fluids (MWF) is the class of oils and liquids used to lubricate, reduce heat and friction between the tool and the part, helping the machining of parts at the time of cutting. The water and nutrients present in the formulation are considered the main sources of microbial contamination. Contamination by microorganisms can affect the health of the operator and also the life of the fluid. The objective of this study was to monitor the microbiological conditions of metal cutting in seven central circulation systems of a metallurgical plant. Monitoring was realized weekly for seven months. The MWF samples were analyzed for concentrations of biocidal actives: N-Butyl-1,2-benzisothiazoline-3-one (NBBIT) and 1,2-benzisothiazoline-3-one (BIT) and counting of microorganisms by pour plating in specific mediums for total mesophilic aerobic bacteria, yeasts and filamentous fungi. All central circulations systems were contaminated by bacteria and yeasts, but not contaminated by filament fungi. The concentrations of biocidal active NBBIT and BIT (200 ppm to NBBIT and 2.500 ppm to BIT) were below those mentioned in the literature as efficient in the control of contamination. Thus, it was concluded that the analyzed metallurgy is not following the dosage of the active biocides which can be harmful to the health of the operators and the quality of the fluid. It is suggested that the company take preventive action measures, such as weekly monitoring and corrective actions such as plant audits to detect and control the microbiological load of the central circulation system.

Keywords: Bacteria. Yeast. Sulfate Reducing Bacteria.

AVALIAÇÃO DA CONTAMINAÇÃO MICROBIANA EM FLUÍDOS DE CORTE DE METAIS EM INDUSTRIA METALURGICA BRASILEIRA

RESUMO: Fluidos de corte de metais (MWF) é uma classe de óleos utilizados como lubrificantes, redutores de calor e atrito entre a ferramenta e a peça usinada. A água e os nutrientes presentes na formulação são considerados as principais fontes de contaminação microbiana. A contaminação por microrganismos pode afetar a saúde do operador e a vida útil do fluido. O objetivo deste estudo foi monitorar a contaminação microbiana em sete centrais de circulação de uma metalúrgica. O monitoramento foi realizado semanalmente durante sete meses. As amostras de MWF foram analisadas quanto às concentrações dos biocidas ativos: N-Butil-1,2-benzisotiazolina-3-ona (NBBIT) e 1,2-benzisotiazolina-3-ona (BIT) e contagem de microrganismos em meios específicos para bactérias aeróbias mesófilas totais, leveduras e fungos filamentosos. Todos as centrais de circulação estavam contaminadas por bactérias e leveduras, no entanto não foram detectados fungos filamentosos. As concentrações dos biocidas NBBIT e BIT ficaram abaixo da recomendada na literatura (200 ppm para NBBIT e 2.500 ppm para BIT). Assim, concluiu-se que a metalúrgica analisada não estava seguindo a dosagem dos biocidas ativos estabelecidos e isso poderá

comprometer a qualidade do fluido e a saúde dos operadores. Sugere-se que a empresa adote medidas preventivas, como monitoramento semanal e ações corretivas, como auditorias na planta para detectar e controlar a carga microbiológica do sistema central de circulações.

Palavras-chave: Bactéria. Levedura, Bactéria Redutora de Sulfato

1 INTRODUCTION

Metalworking fluids (MWF) are used in industrial milling processes of metal parts, can be mixed with water or oil (KAPPOR *et al*, 2014). MWF mixed with water can be divided into water emulsions (> 60 % of concentrated mineral oil); semi-synthetic fluid (emulsions containing 5 to 60 % mineral oil) and synthetic (true fluids or dispersions with <5 % of concentrated oil (SIMPSON *et al*, 2003).

MWF has, as purpose, to help cutting the parts in the machining, milling, reducing the heat and friction between the tool and the part being produced; dissipating and conducting the heat generated through its lubricating and cooling properties. These fluids are of high relevance to maintain the integrity of the surface in the metalworking, protecting against oxidation being used in the process where there are operations in metals such as machining, grinding, deformation impact, abrasive blasting, and polishing (BRINKSMEIER; LUCCA; WALTER, 2004; OECD, 2011).

In the world, 2,000,000 m³ of concentrated MWF is used annually. This volume can be much higher after being diluted in water. After losing its properties, this residue must be disposed of properly and the cost can vary from 28 to 56 U$/m^3. In small businesses, this cost is higher (56 to 113 U$/m^3) (McADAM *et al*, 2012).

Microorganisms use the organic components and additives present in the formulation of fluids as food (OSHA, 2011). Almost all components of MWF can be metabolized by microorganisms as a source of carbon and energy (SEIDEL *et al,* 2017).

The microbiological contamination effects and dramatically reduces the useful life of the fluid since the degradation occurs in an accelerated way generating significant economic losses when the fluid loses its characteristics and has to be frequently replaced (THEAKER; THOMPSON, 2010). The presence of microorganisms in MWF causes significant economic losses. This microbial contamination can lead to the reduction of lubricating and anticorrosive properties (TRAFNY *et al*, 2015).

Besides the loss of MWF's useful life and possibly damage the parts in contact with it, these microorganisms may be associated with the transmission of diseases such as hypersensitivity pneumonia in workers exposed to MWF aerosol during the machining process (NIOSH, 2015).

Operator's exposure to MWF may happen by skin contact and aerosols inhalation (SIMPSON *et al,* 2003). Aerosols may contain all contaminants present in the fluid and contaminate the entire manufacturing extension to where the aerosol can reach. About 1.2 million metallurgical workers within all machining operations are potentially exposed to fluids by aerosols generated in the

machining process when breathing, or by skin contact when handling parts, tools, and equipment treated with fluids (CDC, 2002). Virji et al. (2000) analyzed MWF aerosol samples and observed bacterial counts between $5x10^4$ to $5x10^5$ (colony-forming unit CFU/mL).

However, despite the economic, environmental, operational, and operators health problems, microbial monitoring in plants that use MWF is not routinely performed (TRAFNY *et al,* 2015).

The growth of bacteria and yeast in water-based MWF is inevitable. Thus, biocides should be used to reduce the effects caused by microorganisms (MEYER *et al*, 2017).

The addition of biocidal assets is the most common way to avoid contamination or to control microbial growth in MWF (SEIDEL et al., 2017). The biocidal active 1,2-Benzisothiazolin-3-one (BIT) is used to preserve several products due to its stability to heat, non-volatility, compatibility with ionic and non-ionic compounds, remaining active in acid and alkaline media, and can be used in products that in some step of the process may have contact with heat, without changing its biocidal properties. For some genera of microorganisms such as Pseudomonas and some species of fungi such as *Alternaria alternata* and *Rhodotorula rubra*, the concentrations of the biocide should be higher (up to 2,500 ppm) than those recommended for other species of microorganisms (PAULUS, 2004).

N-Butyl-1,2-benzisothiazoline-3-one (NBBIT) is a biocidal active that exhibits high chemical and thermal stability. MWF emulsions must contain from 50 ppm to 200 ppm of NBBIT. This biocide has shown efficiency against fungi, yeasts, and bacteria. However, for the control of Pseudomonas, concentrations higher than 200 ppm are recommended (PAULUS, 2004).

In this way, companies must monitor the presence of microorganisms as well as concentrations of biocidal assets. Therefore, the objective of this work was to monitor a metallurgical company regarding the count of total mesophilic aerobic bacteria, yeasts, filamentous fungi, presence of sulfate-reducing bacteria (SRB) and concentration of biocidal assets.

2 METHODOLOGY

2.1 SAMPLE COLLECT

The semi-synthetic MWF emulsion samples were collected weekly, sampled period of 7 months (from 02 February to 30 August 2016), in tanks of 7 distribution centers containing approximately 150 m^3 in metallurgical parts for vehicle engines in the interior of the state of São Paulo. The MWF sampled was stored in flasks of 50 mL 20-25ºC.

Samples were analyzed for pH, counts of total mesophilic aerobic bacteria, filamentous fungi, yeast, presence of sulfate-reducing bacteria (SRB), and concentration of biocidal assets.

2.2 DETERMINATION OF MICROBIOTA IN MWF SAMPLES

The plate counting method is most commonly used to monitor MWF (triplicate).

For the microbial count, the MWF samples were diluted in saline solution (0.85% NaCl) and plated in depth using the TSA culture medium (tryptone soybean agar - Difco™ USA) for total aerobic mesophilic bacteria and culture medium SDA (Sabatoud dextrose agar - Difco™ USA) for fungi and yeasts.

The plates for bacterial determination were incubated at 35 ± 2 °C for a period of 48 hours and the yeast and fungal counting plates were incubated at 25 ± 2 °C for a period of 7 days and analyzed after 3-5 days for yeasts growth.

The presence of SRB was determined during the period from March 2 to April 19, 2016, as these may release hydrogen sulfide (H_2S) and cause biocorrosion of pipes and tanks (VIDELA *et al,* 1992).

In 10 mL antibiotic flasks were added 9 mL of Hydrogen Sulphide, Indol, and Mobility medium (SIM), plus 1 mL of MFW sample. The flasks were closed with a butyl cap and sealed with an aluminum cap. In this medium, ferrous citrate and sodium thiosulphate are used to detect H_2S production, the presence of SRB being verified by the blackening of the medium.

The positive control was done with the inoculation of *Desulfovibrio desulfuricans* ATCC 7757, known as sulfate reducer.

The flasks were incubated for a period of 21 days in an oven at 35ºC (± 2ºC).

2.3 BACTERIAL DIVERSITY IN MWF BY DGGE

Fluid samples (central machining 1, 3, and 7 in the months of March and April) were submitted to DNA extraction. Total genomic DNA was extracted with the PowerLyzer® PowerSoil® DNA Isolation Kit (DI MAIRUTA; RÜFENACHT; KÜENZI, 2017) according to the manufacturer's protocol (MOBIO Laboratories, Inc., USA). Fragments of the gene RNAr 16S were amplified by the polymerase chain reaction (PCR) technique using the synthetic oligonucleotides 954f (5´- GCA CAA GCG GTG GAG CAT GTG G- 3´) and 1369r (5´-GCC CGG GAA CGT ATT CAC CG- 3´) for bacteria (YU; MORRISON, 2004).

Amplification was performed in a thermal cycler (Eppendorf) under the following conditions: initial denaturation 94 °C for 5 min; followed by denaturation 1.30 min at 94 °C, annealing for 45s at 38 °C, extension 1 min at 72 °C, and final extension for 5 min at 72 °C. PCR negative control was performed with all components of the PCR reaction except DNA.

At the end of the PCR, the samples were applied in agarose gel electrophoresis, (80 V for 60 minutes with the 1 kb marker). The DNA bands were visualized in a transluminator with UV light and then the visualization of the bacterial diversity of the sample was developed by Denaturing Gradient Gel Electrophoresis (DGGE).

The DDGE was made in the DCode system equipment (BIO-Rad, USA), according to Rosas et al. (2004). The gel was made from 45% to 65% denaturing gradient polyacrylamide prepared from stock solutions of polyacrylamide (6%), one containing 0% and one containing 100% denaturing agents (7M of urea 100% and 40% deionized formamide). It took 3 hours for polymerization.

The electrophoresis was developed at 60 °C, 60 V, for 18 hours. After the run, the gel was stained with silver nitrate. For staining, the gel was shaken for 30 min in fixative solution (50 mL ethanol, 2.5 mL glacial acetic acid and filled to 500 mL distilled water), washed with distilled water, stirred 30 min in silver nitrate solution (0.48 g of $AgNO_3$ and completed to 300 mL of distilled water), then developer solution (7.5 M NaOH, formaldehyde 2.25 mL, completed to 300 mL) was placed, upon reaching the desired color, was placed a stop solution (15 mL glacial acetic acid and completed to 50 mL). The gel was washed in distilled water and photographed.

DGGE band profiles were analyzed using Bionumerics software version 3.4 (Applied Maths, Belgium). Similarity calculations were based on the Pearson correlation coefficient. Through these calculations, cluster analyzes were developed to form dendrograms (RECHE et al., 2005).

2.4 DETERMINATION OF BIOCIDES (NBBIT E BIT)

All the collected samples were evaluated for the concentration of biocidal assets present. Assay determination was performed, weighing 0.5 g of the collected fluid sample in duplicate. To prepare the solution, 10 g of HPLC grade acetonitrile and 0.25 mL of acetic acid were added, stirred and the supernatant was collected by filtering with a membrane of 0.22 µm. Stock solutions of biocidal assets were prepared to construct the standard curve using the biocidal assets 1,2-benzisothiazoline-3-one (BIT) (LONZA a, 2013) diluted with HPLC grade acetonitrile and acetic acid until complete dissolution. To prepare the N-Butyl-1,2-benzisothiazoline-3-one (NBBIT) standard, 500 ppm of the biocidal asset was diluted in methanol (LONZA b, 2013).

The chromatograph HPLC Waters 2695 Model Alliance was calibrated with UV detector 2489 operating at 318 nm and Symmetry C18 5µm 4.6x250 mm column for BIT mobile phase water: acetonitrile: methanol: acetic acid (68:17:12:3) at 30 °C and for NBBIT mobile phase water: acetonitrile: methanol: acetic acid (65:25:20:2.5) at 40 °C both for about 30 minutes and it was injected with the standards and samples.

For the calculations, the chromatograms obtained with the standards and with the samples were compared. Peaks that presented satisfactory resolutions regarding height, shape and retention time were integrated into the chromatogram, and evaluated through the software installed in the computer.

3 RESULTS AND DISCUSSION

The semi-synthetic emulsion fluids evaluated were diluted to the recommended minimum concentration of 8%, indicated for centralized systems, and distributed in individual machines, for general machining of cast iron, carbon steels, alloy, and stainless steels, aluminum and its alloys, and yellow metals. The complete composition of the fluid could not be determined.

The pH of the central were monitored throughout the experiment, remaining in the range of 8.1 to 9.0.

Throughout the monitoring, the concentration of the biocidal assets NBBIT and BIT were evaluated for the preservation of the semisynthetic fluid emulsion.

The biocidal assets, BIT and NBBIT, used in the preservation of the emulsions during this monitoring were indicated by the manufacturer because they are compatible with the characteristics of the MWF and the machined parts.

BIT concentrations were also below that recommended by the manufacturer, however, in the fourth week of the central 4 samplings, the concentration of its asset was at 80 ppm, being the only sample within the recommendations.

The NBBIT concentrations of the analyzed central, were below the recommended by the manufacturer and were observed differences during the evaluated weeks and central. For NBBIT, the lowest concentrations were observed in the central 7 (<2 to 5 ppm) and in this same central was observed the presence of SRB in all the weeks sampled.

Paulus (2004) described that NBBIT concentrations required to control the main microorganisms found in MWF should be in the concentration up to 2,500 ppm and for BIT between 50 and 200 ppm.

According to Trafny et al. (2015), the presence of biocides did not reduce the count of bacteria in the analyzed factories, probably due to the biofilm resistance. These authors verified the formation of biofilms in several machining machines and pointed out that the presence of biocide may cause greater environmental stress for the bacterial community.

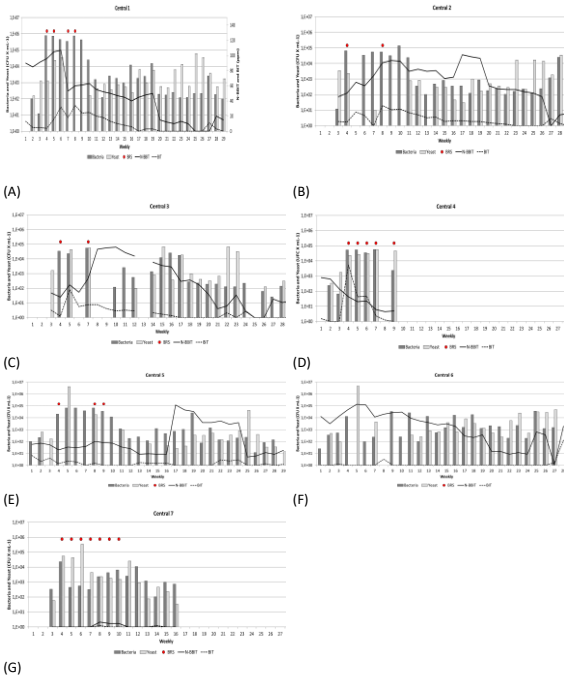
Figures 1 present the results of total mesophilic bacteria counting and yeasts (CFU/mL) and active concentrations in ppm, in the period of 29 weeks for each central evaluation. The data of filamentous fungi were not included because there was no growth in any analyzed sample. However, fungi such as *Fusarium* spp., *Exophiala* spp., *Trichoderma* spp. and *Penicillium* spp. have already been found in MWF (TRAFNY et al., 2015; KAPOOR et al., 2014)

During the monitored period, the MWF emulsions showed a great variation in the count of microorganisms, with a maximum of 10^5 CFU /mL.

For machining central 1, the count of microorganisms after the 3rd week of monitoring was up to 10^5 CFU/mL. Most weeks had 10^2 CFU/mL for counts of bacteria and yeasts, with frequencies of 41 and 44%, respectively. For SRB, the presence was observed in 57% of the samples. The concentrations of NBBIT assets ranged from < 2 to 107 ppm, and BIT was < 2 to 34 ppm (Figure 1A).

Machining central 2 was monitored for 27 weeks, and the samples showed 55% had counts of 10^2 CFU/mL, but the contamination reached up to 10^5 CFU/mL. For yeasts, the maximum count was 10^4 CFU/mL in 18% of the samples. For SRB, this central had lower contamination, with 28% of samples having positive results. For the biocidal assets, these ranged from < 2 to 98 ppm for NBBIT and < 2 to 10 ppm for BIT (Figure 1B).

Figure 1 - Counting of yeasts and total mesophilic aerobic bacteria (CFU), presence of SRB, and concentration of biocidal assets (ppm) in cutting fluid for metals - machining central.



At the machining central 3, it was monitored for 26 weeks, the contamination was for the entire period below 10^5 CFU/mL, with 42% of the samples with counts of 10^2 CFU/mL. For yeasts, the counts were below 10^5 CFU/mL, with 34% of the samples having counts of 10^2 CFU/mL. This central had the same percentage of SRB presence as central 2. The variation of NBBIT assets was < 2 to 103 ppm and BIT of < 2 to 41 ppm (Figure 1C).

The machining central 4 was monitored only for 9 weeks due to machining stoppage, no counts above 10^4 CFU/mL were found for bacteria, for yeasts, this value was observed in 55% of the samples and 44% for bacteria, SRB was present in 71% of the samples. Concentrations of NBBIT ranged from 14 to 35 ppm and BIT varied from < 2 to 80 ppm (Figure 1D).

Machining central 5 was monitored for 29 weeks and had counts of up to 10^4 CFU/mL in 27% of bacterial samples, with a higher count of 10^2 CFU/mL (37%). SRB were present in 42% of samples. For yeasts, a single count of 10^6 CFU/mL was observed. For the remaining weeks, the counts were 10^2 CFU/mL (34%). This central had the lowest concentration of NBBIT of 15 ppm (the highest for all Centrals) and a maximum of 109 ppm, but for BIT active were observed concentrations of < 2 to 10 ppm (Figure !E).

The machining central 6 was monitored for 29 weeks with counts below 10^5 CFU/mL for bacteria and showed the highest similarities between the counts in the period, with 31% of the samples with 10^3 CFU/mL. Yeast count of 10^6 CFU/mL was observed in a sample of this central, in the other weeks, 31% of counts were observed with 10^2 CFU/mL. No SRB was found in any sample of this central. The maximum concentration of NBBIT found in all monitoring analyzes was observed in this central with 110 ppm and the BIT active variation was < 2 to 11 ppm (Figure 1F).

At machining central 7 was monitored for 14 weeks due to the central shutdown. Total bacterial counts were below 10^5 CFU/mL, and contamination of 50% of samples at 10^2 CFU/mL. Although the counts were lower than in the other centrals, the presence of SRB was in 100% of the samples. The yeast counts were up to 10^5 CFU/mL in only one sample, in the others the counts were close to 10^2 to 10^4 CFU/mL. In this central, were observed the lowest concentration of the biocidal assets

NBBIT (< 2 to 5 ppm) and BIT (< 2 to 2 ppm) (Figure 1G).

Central 6 had the highest concentrations of NBBIT with a maximum of 110 ppm. In this central, the presence of SRB was not detected in the monitoring period and the NBBIT concentrations were between 87 - 110 ppm, being the highest in the period. Despite this finding, it was not possible to find a direct relationship between of the assets concentrations and the presence of SRB in the central. The SRB presents several species being most anaerobic.

The presence of SRB may be associated with corrosion and the formation of biofilms. SRB of the *Citrobacter* was isolated from MWF emulsions (Zhang et al., 2015). Counts of bacteria close to those obtained in the present study were reported by Khan; Yadav (2004) in 20 samples of MWF (10^4 to 10^5 CFU/mL)

Di Mairuta; Rüfenacht; Küenzi (2017) stated that although the microorganisms counting in plaque be the most used method, the results obtained may be underestimated as much as the density as in diversity. Some microorganisms may not grow under the imposed conditions. The authors used massive DNA sequencing and observed the predominance of *Pseudomonas* in all samples.

Lodders; Kampfer (2012) demonstrated in their study that the potential contamination of an emulsion can reach 10^8 UFC/mL for bacteria. Salmeen et al. (1987) reported larger counts reaching up to 10^9 CFU/mL.

Kapoor et al. (2014) obtained bacterial counts of 10^7 to 10^{10} CFU/mL and observed that the cleaning was not able to eliminate the contamination, there is probably biofilm formation in the system and these were not removed during the cleaning procedure. The addition of biocide did not affect immediately the reduction of bacterial counts, thus indicating that it had a bacteriostatic effect.

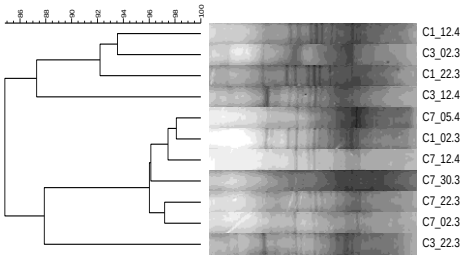
Trafny et al. (2015) evaluated the bacterial count in 10 industrial plants in Poland and found that 30% of these exceeded the count of 10^6 CFU/mL and that in some machining machines it reached 10^8 CFU/mL.

The samples selected for PCR/ DGGE analysis were: 03 samples from Central 1 (C1), 03 samples from Central 3 (C3), and 05 samples from Central 7 (C7). This selection was random, however, it involved samples with presence and absence of SRB, higher concentrations of NBBIT biocidal assets, and different counts for bacteria and yeasts.

The DGGE bands are presented in a dendrogram using the Pearson correlation (Figure 2). It was not possible to equate the presence of specific bands by genus or species, however, a low number of bands (less than 20) was observed when compared to soil or biological sludge samples. This low diversity may be a reflection of the selective nature of MWF (VAN DER GAST *et al*, 2003) and the presence of biocidal assets. A high similarity (greater than 86%) was observed among all analyzed samples. Higher similarities were observed between the centrals C1-02.03 and C7 05.04 (98%) and C7-22.03 and C7-02.03 (97%). This high similarity found in all analyzed samples indicates that the bacterial community is very similar due to the operating conditions such as pH, temperature, MWF, biocides used.

It was not possible to establish a correlation between the band profiles and the microbial counts and the concentration of biocides.

Figure 2 - Grouping (UPGMA) of DGGE band profiles using Pearson correlation for MWF bacterial communities of machining centers (C) 1, 3, and 7 central machinings ondiversity different collection days collect dates



C1= central machining 1
C3= central machining 3
C7= central machining 7
The other numbers are from the collection dates

Van der Gast *et al*. (2003) analyzed the MWF samples employing DGGE and observed low bacterial diversity (number of bands smaller than 10).

Trafny et al. (2015) attributed that some bacteria present in the MWF may not grow in used solid medium and thus not detected in the count. They observed that there was a greater diversity of bacteria in MWF with biocides and that this may be an important factor in the functional preservation of the bacterial community in hostile environmental conditions.

According to Kapoor et al. (2014) independent molecular methods of cultivation such as DGGE combined with other methods of identification may be used in MWF since it is necessary to understand both cultivable and non-cultivable fractions; as well as the structure of the total microbial community. The authors evaluated synthetic MWF after recharging and cleaning for 65 weeks. The bacterial counts were quite high and observed even after cleaning. The PCR confirmed the prevalence of 6 potentially pathogenic groups: pseudomonad, enteric, mycobacteria, legionellae, actinomycetes, and fungi. DGGE revealed a bacterial richness of 23 bands per sample. According to Van der Gast et al. (2002) the MWF exerts a selective pressure, which favors different populations of bacteria and this way low diversity.

The work of Di Mairuta, Rüfenacht; Kuenzi (2017) was compared microbial diversity MWF with and without biocides. Microbial diversity was very similar, but not identical.

4 CONCLUSIONS

The monitoring performed can demonstrate the susceptibility of the cutting fluids and their great potential of microorganisms proliferation.

It was found that the concentrations of biocides present in the MWF were below that indicated in the literature and this may have contributed to the growth of total aerobic bacteria and sulfate-reducing bacteria such as yeast. The present study does not detract from the quality of the biocides used in the company, on the contrary, it recommends greater attention to the dosages of the biocidal additives so that they can control the microorganisms. It is considered premature to state that the use of biocides below the

recommendation may contribute to microorganism resistance.

The presence of SBR in most plants indicates the potential for biocorrosion.

Bacterial diversity appears to be very similar between centrals.

It is also recommended to check the formation of biofilms in machines and piping and that periodic cleaning is done on the machines and also close to the machining site to reduce contamination.

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