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Assessment of flow cytometry for microbial water quality monitoring in cooling tower water and oxidizing biocide treatment efficiency

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ABSTRACT

The control of *Legionella* proliferation in cooling tower water circuits requires regular monitoring of water contamination and effective disinfection procedures. In this study, flow cytometry was assessed to monitor water contamination and disinfection treatment efficiency on bacterial cells regarding nucleic acid injury (SYBR® Green II), cell integrity (SYBR® Green II and propidium iodide) and metabolism activity (ChemChrome V6). A total of 27 cooling tower water samples were analyzed in order to assess water contamination levels regarding viable populations: standard culture, ATP measurement and flow cytometry methods were compared. Flow cytometry and plate counts methods showed a significant correlation for changes in concentrations despite a 1 to 2-log difference regarding absolute quantification. Concerning intracellular activity, the use of two different flow cytometers (FACSCanto™ II and Accuri™ C6) showed no statistical difference while a difference was observed between flow cytometry and usual methods (culture and ATP measurement). The standard culture and flow cytometry methods were also compared for *in vitro* bacteria inactivation measurements in the presence of 3 different types of oxidizing biocides commonly used for cooling tower disinfection. Reductions observed ranged between 1 and 2 log depending on (1) the detection method, (2) the bacterial population origin and/or (3) the active biocide molecule used. In conclusion, flow cytometry represents an efficient, accurate and fast approach to monitor water contamination and biocide treatment efficiency in cooling towers.

1. Introduction

The conditions encountered in cooling tower systems are ideal for *Legionella* development and proliferation in water circuits and high proportions of occurrence are reported (Li et al., 2015; Rafiee et al., 2014). During this process, water droplets may release these pathogenic bacteria to the surroundings and can lead to human exposure and thus to outbreaks of legionellosis (Ambrose et al., 2014; Lévesque et al., 2014; Scaturro et al., 2015). In France, the management and monitoring of cooling towers as part of *Legionella* risk prevention is regulated, specifying characteristics of installations subject to declaration or registration. Common methods for environmental monitoring of *Legionella* are the standard culture-based method (NF T90 431, 2017). Health risk management is based on *Legionella* investigation to verify that the level remains below alert thresholds. Indeed, a level greater than or equal to 10^3 colony forming units per liter (CFU l^{-1}) must lead to risk analysis and corrective actions; a level higher or equal to 10^5 CFU l^{-1} imposes installation emptying, cleaning and disinfection. Chemical biocide control in cooling towers is a critical part of the overall prevention strategy against *Legionella*. Various biocide treatment programs, by volumetric injection or regulated and alternating shocks are commonly used to limit microbial growth in the systems. As a result, count of culturable microorganisms (ISO 6222, 1999) is used as bacterial indicator related to the biocide treatment effectiveness. The advantage of such monitoring lies on the fact that the quantification of the total culturable flora is easier and faster (48 h) than *Legionella* detection, showing a time to result of > 10 days. However, this method has also some drawbacks in terms of active risk management. Even if the time to result is shorter than for *Legionella*, a 2-day period is still long. Furthermore, this culture-based method enables to visualize only the microbial cells able to grow on a culture medium. In fact, < 1% of bacteria found in the environment are culturable (Hammes et al., 2008; Wang et al., 2010). Indeed, a major part of microbial population, that does not have or have lost the ability to form colonies on agar plates under stress conditions, is called viable but not culturable (VBNC). These bacteria may nevertheless still have active cellular machinery, which makes possible their potential pathogenicity for humans (Ramamurthy et al., 2014; Zhang et al., 2015). In addition, a culture-

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based method requires a sample to be sent to the laboratory and cannot be performed directly in the field. The ATP measurement is also frequently used in the field for cooling tower disinfection monitoring (Duda et al., 2015; Mueller et al., 2009) and gives information on global microbial activity. However, this method does not provide precise data related to the real bacterial concentration and to the impact of biocidal treatments employed.

Flow cytometry is an alternative technique enabling to overcome the aforementioned constraints. Its principle allows individual qualitative and quantitative characterization of cells suspended in a liquid medium. Besides to a rapid quantification, flow cytometry provides in a short time information related the physiological state of cells through the use of adapted fluorochromes and has been adapted to different matrices such as seawater and drinking water (Grégori et al., 2001; Hammes and Egli, 2010; Helmi et al., 2014). However, few studies have been carried out using flow cytometry in the field of disinfection and *Legionella* issues in cooling tower (Fuchslin et al., 2010; Keserue et al., 2012). Moreover, these works have been mainly conducted using only pure *Legionella* strains. The detection approaches are based on the use of antibodies to obtain a specific detection (Fuchslin et al., 2010; Tyndall et al., 1985) or even to demonstrate their viability using fluoregenic compounds (Keserue et al., 2012). These approaches present considerable cost in terms of consumables due to the use of antibodies for immunomagnetic separation and for specific detection and require some expertise in sample preparation. In view of these observations, these methods are difficult to apply in real conditions. Moreover, these approaches do not provide information related to the impact of disinfection on the entire microbial population, potentially having the ability to generate a biofilm. Other tests in order to evaluate the impact of different biocidal treatments have been also carried out directly on pure *Legionella* cultures, namely without the use of antibodies, using double staining with SYTO9 and PI (Allegra et al., 2008; Mustapha et al., 2015). A team studied the resistance of different strains of *Legionella* to chlorine dioxide, ClO_2, (Mustapha et al., 2015). The authors stated that switch to the VBNC stage, measured using flow cytometry, takes place between 4 and 5 $mg\,l^{-1}$ of ClO_2. The VBNC status can be reversible and some VBNC cells can regain their ability to grow afterwards. Another study (Allegra et al., 2008) demonstrated that *Legionella* cells can lose their ability to grow after heat treatment and then recover it by passing an intermediate VBNC state that is not detectable by conventional cultivation methods. This point emphasizes the fact that the measurement of VBNC cells is an important aspect in terms of safety.

The present work has been carried out on the quantification of active microbial cells, a category that may include VBNC and culturable bacteria, irrespective of genus or species. Compared with the approaches described above, the advantages are related to the fact that the measurement of the impact of a biocidal treatment is evaluated on the whole natural indigenous population, which may behave differently from pure strains. The relevance of flow cytometry has been investigated in order to highlight the gain related to cooling tower monitoring compared to usual methods and to define to what extent the performance would allow to adapt biocide treatment rates. For this purpose, the first part of the study was dedicated to real cooling tower water sample analysis to compare the detection performance of flow cytometry, ATP measurements, and the standard culture-based method. The second part of the study was based on a kinetic assessment in order to compare biocide way of action on cooling tower indigenous flora.

2. Materials and methods

2.1. Sample collection

A total of 27 tertiary cooling tower samples were collected over a 4-month period (from 20 different sites). Water was sampled in 1-l plastic bottles containing 20 mg of thiosulfate and delivered the same day to the laboratory. Samples were kept at 4 °C until analysis, including flow cytometry and culture methods, with the addition of ATP measurement only for the last 10 samples due to collection limitation. Free chlorine potentially remaining after neutralization was measured in the samples using the DPD colorimetric method (Hach, USA). In addition, water from two different cooling towers was collected to prepare bacterial suspensions for assessment of biocide impact on cells. The first sample was from a cooling tower fed with potable water (tertiary) and a second sample was collected from a cooling tower fed with fresh water (industrial). Microbial population isolated form each water origin were respectively entitled population 1 (from potable water sample) and population 2 (from fresh river water sample).

2.2. Flow cytometry

Experiments were performed with FACSCanto™ II (laboratory system) and Accuri™ C6 (fieldable system) flow cytometers (Becton Dickinson, California, USA). In order to compare both system performance, the assays related to real sample analysis were performed using simultaneously the two cytometers. The assays dealing with biocide impact assessment were performed using only FACSCanto™ II. The applied protocol enabled to discriminate between live and dead or active and inactive bacterial cells with approaches based either on membrane integrity or metabolic activity. To distinguish intact and damaged bacterial cells, SYBR® Green II (SGII, Molecular Probes, Oregon, USA) and propidium iodide (PI, Sigma Chemical Co.) were used simultaneously as permeant and impermeant dyes, respectively. Briefly, 10 µl of stock solution of PI (10 $mg\,ml^{-1}$) and 10 µl of a 1-log diluted SGII commercial solution in dimethyl sulfoxide (DMSO) were added simultaneously to 100 µl of sample (1-ml final volume). Samples were then incubated for 20 min at room temperature in the dark. To differentiate active bacteria, the esterase activity was highlighted using ChemChrome V6 (CV6, bioMérieux, France). Briefly, 100 µl of each sample were added to 890 µl of Chemsol B16 buffer (bioMérieux, France) and supplemented with 10 µl of CV6. Samples were incubated for 30 min at 30 °C in the dark and were analyzed directly after incubation to avoid excessive background noise. In addition, a control without staining was systematically performed in parallel for each sample, in order to quantify autofluorescent particles.

2.3. Culture methods

Regarding culturable bacteria, heterotrophic plate count (HPC) method was applied according to the standard (ISO 6222, 1999). Briefly, 1 ml of the appropriate dilution was transferred into a sterile Petri dish and mixed in about 20 ml of PCA (Plate Count Agar) media. Colonies were enumerated after 48 h of incubation at 36 °C, using a colony counter (Scan® 1200, Interscience, France). All culturable counts were expressed as CFU ml^{-1} (Colony Forming Units per milliliter). Detection of *Legionella* spp. and *Legionella pneumophila* was performed according to the standard protocol NF T90–431. The procedure was suitable for samples with high turbidity, namely using filtration of 500 ml, followed by sonication of the polycarbonate membrane for resuspension, prior deposit on the culture medium. Briefly, the method relies on two successive steps including a first growth using the selective nutrient medium GVPC then a second growth using BCYE supplemented or not with cysteine, and incubation for 10 days at 37 °C. Typical colonies were enumerated after three, five and ten days of incubation and *Legionella pneumophila* species were identified *via* a latex agglutination test (Microscreen).

2.4. ATP measurement

ATP measurement was performed (triplicates), using the QGA™ kit Quench-Gone Aqueous (Aquatools, France). Briefly, 25 ml of sample was concentrated through a 0.22-µm filter and bacteria were lysed to

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