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APPLICATION OF A DISRUPTIVE METHOD TO MITIGATE HEALTH AND MICROBIOLOGICAL RISKS IN POWER PLANT COOLING WATER CIRCUITS

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ABSTRACT

Water dispersion cooling systems in an air flow used in cooling towers and sanitary hot water systems generate health and microbiological risks due to the presence of *Legionella* and *Amoebae*.

These risks are considered by the operators and authorities, through control strategies and treatment of incriminated circuits. However, conventional *Legionella* quantification methods for these types of water systems are likely to provide highly variable results from one laboratory to another, therefore leading to inconsistent actions relative to the actual situation.

This variability was highlighted by a comparison with the NuMICA *Legionella* solution, recently developed by Diamidex, a French company dedicated to the microbiological control, in partnership with NUVIA, a company which offers services to nuclear facility operators.

1. BACKGROUND

Cooling systems such as cooling towers used in power plants and sanitary hot water systems generate health and microbiological risks by providing favorable development conditions for microorganisms such as *Legionella* and *Amoebae*. Thus, in most industrialized countries, operators using water dispersion cooling systems in an air flow are required to ensure the health and microbiological safety of installations through a risk analysis approach, which is based on monitoring of changes in sanitary and microbiological quality, and which aims to control the proliferations of pathogenic agents.

Various organizations and authorities worldwide require from the operators (or highly recommend) to carry out controls as well as to take adequate measures not to exceed certain thresholds, under penalty of initiating unfavorable actions. Operators then implement control strategies defined by detection frequencies, detection method(s), and appropriate measures to manage the risks. Those measures generally include thermal and chemical treatments, which should be adjusted to the level of contamination.

2. PROBLEM STATEMENT

1) *Legionella* and legionellosis

"Legionella was discovered after an outbreak in 1976, among people who went to a Philadelphia convention of the American Legion. Those who were affected suffered from a type of pneumonia that became known as "Legionnaires' disease" (Center for Disease Control and Prevention, 2022). Infection occurs after inhalation of contaminated water aerosols. *Legionella* is a genus of bacteria which is naturally present in hydro telluric environments, and which proliferates nowadays in many artificial environments associated with human activity due to appropriate conditions for their growth, such as temperature ranging from 25°C to 50°C, water stagnation, presence of corrosion, scale deposit, biofilms, etc. *Legionella* includes 49 species of bacteria. Among these, *Legionella pneumophila* (figure 2.1) is a pathogenic species which has been involved in most cases of contamination. This species is itself divided into 16 serogroups, of which serogroups 1, 4 and 6 were described as the most virulent. Nevertheless, this proportion between serogroups can vary, depending on the different geographical locations, the types of facilities and the type of public exposed. For instance, a study (2002) based on 1,335 cases of legionellosis (Legionnaires' disease) caused by *Legionella pneumophila* in Europe showed that at least 36% of these infections contracted in a European hospital involved serogroups of *Legionella pneumophila* other than serogroup 1 (Helbig *et al.*, 2002). This percentage has been varying according to the regions of the world and could reach 71% in Denmark, Finland, and Sweden. Currently, legionellosis is an illness which must be declared in many countries around the world (National Academies of Sciences, Engineering, and Medicine, 2020). According to the World Health Organization (WHO), *Legionella* represents the highest health burden of all waterborne pathogens in the European Union (EU) (WHO, 2006, 2007, 2011, 2017). Between 2011 and 2018, the number of legionellosis cases reported in Europe – the majority of which (69%) in France, Germany, Italy, and Spain – had increased from 1.0 to 2.9 cases per 100,000 inhabitants. In the United States, the Center for Disease Control and Prevention (CDC) estimates that more than 25,000 cases of Legionella-related disease occur each year, causing more than 4,000 deaths. *Legionella pneumophila* has been globally responsible for the majority of legionellosis (80-90%), including serogroup 1 (70-80%), and followed by serogroup 6 particularly in the United States (AWT, 2019; National Academies of Sciences, Engineering, and Medicine 2020).

To deal with the growing increase of legionellosis in industrial countries, the WHO and the EU recommend performing periodic microbiological analysis to detect the presence of *Legionella*, especially in large buildings. These recommendations are intended to improve legislations and/or overcome the lack of regulation(s) in countries.

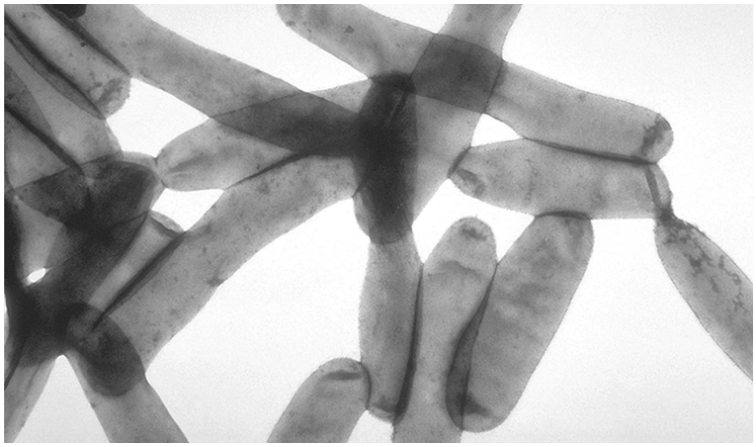


Figure 2.1. *Legionella pneumophila*, electron microscopy (photographic credits: CDC Public Health Image Library).

2) Management of the Legionella risk

Sanitary and microbiological risk control depend on the frequency of controls and the reliability of the detection method since the early detection of a potential risk makes it possible to prevent a problematic situation on the one hand, while the results of microbial count directly affect the type of measure or treatment applied to correct a proliferation on the other hand.

Regarding *Legionella* in cooling water circuits, conventional methods used by laboratories have been found to produce variable results, which is particularly problematic when the measured values are close to the thresholds for corrective actions. Decision-making based on the results is likely not to consider the real risk and therefore to lead to inconsistent actions resulting in consequences that are systematically detrimental to the operators. Indeed, while overestimated results may induce at least unnecessary or excessive treatment, underestimated results lead to a lack of action precisely where a real risk should be taken into consideration. The underestimation of risks is particularly to be feared. In practice, operators tend to apply indiscriminate and massive treatments because they prefer to limit the risks as much as possible rather than having to deal with problems. However, these massive treatments generate non-negligible costs economically, energetically, and environmentally. Hence, the need for rapid and reliable detection methods arises.

In this article, the variability of *Legionella* concentration results produced by the conventional French method NF T90-431:2017 (AFNOR, 2017), being analogous to the international method ISO 11731:2017 (ISO, 2017), was highlighted through comparison with the NuMICA *Legionella* solution, which involves a disruptive control method based on the selective detection of *Legionella* via a specific marker bound by “click chemistry”.

3. MATERIAL AND METHODS

1) Sample preparation

10L samples were prepared. For the simple matrix samples, a known quantity of *Legionella pneumophila* serogroup 1 (environmental strain isolated from a naturally contaminated sample) was added to sanitary hot water, which was previously collected from an uncontaminated network. Each sample was then homogenized and distributed in twenty 500 mL sample bottles to be analyzed (in accordance with NF T90-431 for this type of matrix). For the complex matrix samples, cooling tower water was taken from a naturally contaminated system. Each well-homogenized sample was then distributed into ten 1L sample bottles to be analyzed (in accordance with NF T90-431 for this type of matrix).

2) Sample analysis by using NF T90-431:2017 method

Half of the test portion samples to be analyzed with the NF T90-431 method were sent to COFRAC accredited laboratories: 10 test portion samples for sanitary hot water and 5 for cooling tower water samples. NF T90-431 method is a culture method which involves counting the number of *Legionella* colonies formed on GVPC agar medium from a spreading or filtration of the analyzed sample. Reading of the agar medium dishes must be done manually after 3 to 10 days of culture at 36 +/- 2°C. It is then necessary to carry out an identification test to confirm the colonies which look like *Legionella* colonies and to determine whether they belong to *Legionella pneumophila* species or to another species. For sanitary hot water matrices, the count is made on a 200 µL spread, and 10 mL and 100 mL filtrations on a cellulose ester membrane of 0.45 µm porosity. To limit the growth of interfering flora on the filtration membranes, an acid treatment was applied to the membranes just after filtration. The detection threshold for this type of matrix is 10 CFU/L. For cooling tower water matrices, the count is made on two spreads of 20 µL and 200 µL sample, and on spreads of 100 µL of the sample concentrated 100x by centrifugation or by filtration. In order to limit the

interfering flora on the spreads of the concentrated sample, acid, thermal and combined acid-thermal treatments are carried out. The detection threshold for this type of matrix is 100 CFU/L.

3) Sample analysis by using NuMICA *Legionella* solution

The second half of the test portion samples was analyzed by using NuMICA *Legionella* solution in the Diamidex laboratory, according to the supplier's recommendations. NuMICA *Legionella* solution is also based on culture, supplemented by specific labeling of *Legionella pneumophila* cells. The analysis was carried out on 20 mL of water sample filtered through a PVDF membrane of 0.45 µm porosity. Similarly to the French regulatory method, the membrane was subjected to an acid treatment which makes it possible to limit the interfering flora. The membrane was then laid over a drop of 500 µL of culture supplement (containing the specific marker molecule), itself deposited on a GVPC agar medium. For cooling tower water samples, which are generally heavily loaded with interfering flora, an extra heat treatment was subsequently carried out by incubation at 52°C for 45 minutes. The agar was incubated at 37°C for 48 hours thereafter. After incubation, the membrane was transferred to a fiberglass pad soaked with 700 µL of a labeling solution, then incubated at 30°C for 15 minutes, then washed for 15 minutes on a dedicated washing bench to eliminate the excess labeling solution. Finally, the membrane was scanned by the NuMICA micro-colony counter. The artificial intelligence embedded in the counter specifically identified the *Legionella pneumophila* micro-colonies on the images, and directly returned the concentration in the sample in CFU/L. Under the experimental conditions thus described, the detection threshold set in the NuMICA was 100 CFU/L. This detection threshold depends on the volume of sample filtered and could for example be 10 CFU/L when 200 mL of sanitary hot water sample is filtered.

4) Statistical analysis

To be able to consider and graphically represent negative results, these are replaced by the threshold value of 100 CFU/L (NF T90-431 threshold for cooling tower water samples, and NuMICA threshold). Microbiological counts generally follow a log-normal type distribution. Data were therefore converted to log10 for all statistical analyses. It is generally considered that the measurement uncertainty of microbiological counts is equivalent to ± a half power of 10, or ± 0.5 log (Service d'accréditation Suisse, 2017). It was therefore considered that two results were similar when their difference was less than 0.5 log. For samples for which a theoretical value could be determined (additions of a known quantity of *Legionella pneumophila*), the deviation from this value was calculated by using Equation 1 below:

$$bias = log_{10}(C1) - \log(C2) \quad (1)$$

Where C_1 is the experimental concentration (CFU/L), and C_2 is the theoretical concentration (CFU/L).

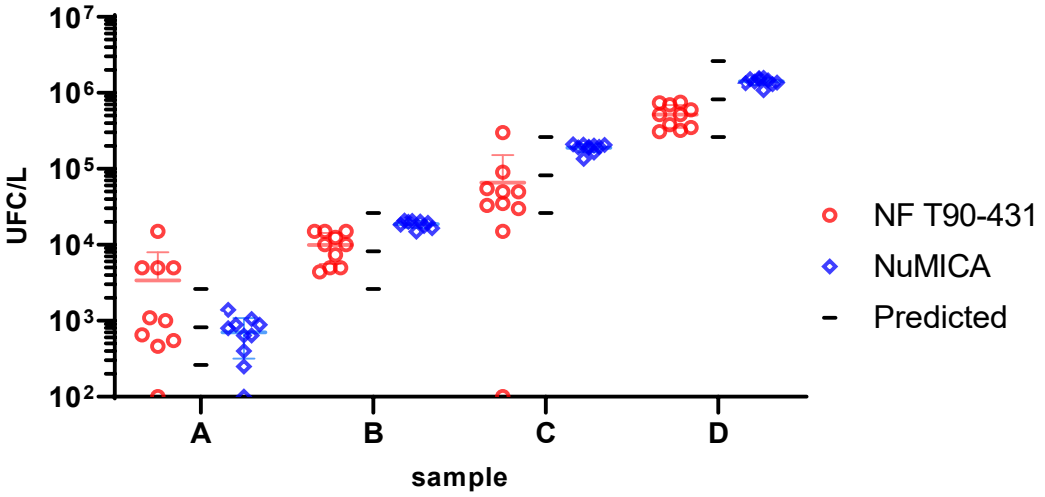
4. RESULTS OF THE COMPARATIVE ANALYSIS

The variability of the NF T90-431 method and NuMICA *Legionella* solution, both based on *Legionella* culture, were assessed by analyzing multiple test portions from the same samples.

1) Sanitary hot water matrices

The study about sanitary hot water matrices was carried out with a dilution range of *Legionella pneumophila* added to the analyzed samples. This allowed to obtain 4 samples A to D containing a *Legionella pneumophila* density estimated at 825, 8,250, 82,500 and 825,000 CFU/L respectively. The graphical representation of the results of the analyzes by the two methods (Figure 4.1) made it possible to show that results obtained with NF T90-431 method were generally more dispersed, for a given sample,

than those obtained with NuMICA *Legionella* solution. It should also be noted that NF T90-431 method produces false negatives even at high density (82,500 CFU/L, sample C).



The graphs present the individual data, and the means and standard deviations of the NF T90-431 method and NuMICA analysis. Predicted values are presented as an expected value (the middle dash of each group) and low and high tolerances around this value, corresponding to a difference of +/- 0.5 log units. The points placed on the X-axis correspond to the negative results reduced to the detection threshold value.

Figure 4.1 Values obtained for sanitary hot water with the two methods.

The average standard deviation of measurements with NF T90-431 method was 0.48 log units, versus 0.12 log units with NuMICA *Legionella* solution. The NuMICA *Legionella* solution displayed high standard deviation principally for samples with very low density, close to the detection threshold of the method, whereas the NF T90-431 method displayed high variability for all densities (Table 4.1). The number of overestimates, like the number of underestimates, was also higher with NF T90-431 method than with NuMICA *Legionella* solution.

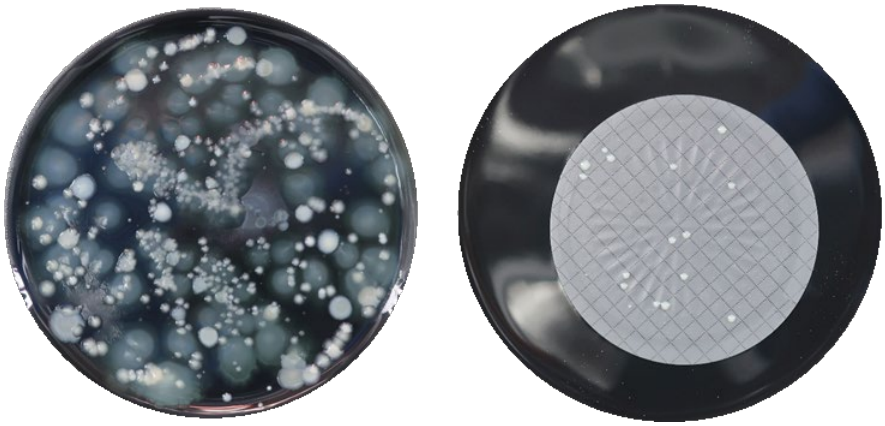
Table 4.1 Summary of the results obtained for sanitary hot water.

	Sample	A	B	C	D	Total
	Theoretical density [log10 (UFC/L)]	2,92	3,92	4,92	5,92	
NF T90-431	Mean [log10 (CFU/L)]	3,15	3,95	4,43	5,69	
	Std deviation	0,65	0,21	0,92	0,15	
	Overestimations*	4	0	1	0	5
	Underestimations*	1	0	2	0	3
NuMICA	Mean [log10 (CFU/L)]	2,76	4,27	5,27	6,15	
	Std deviation	0,34	0,05	0,06	0,05	
	Overestimations*	0	0	0	0	0
	Underestimations*	2	0	0	0	2

** number of results above or below the theoretical density by more than 0,5 log units.*

2) Cooling tower water matrices

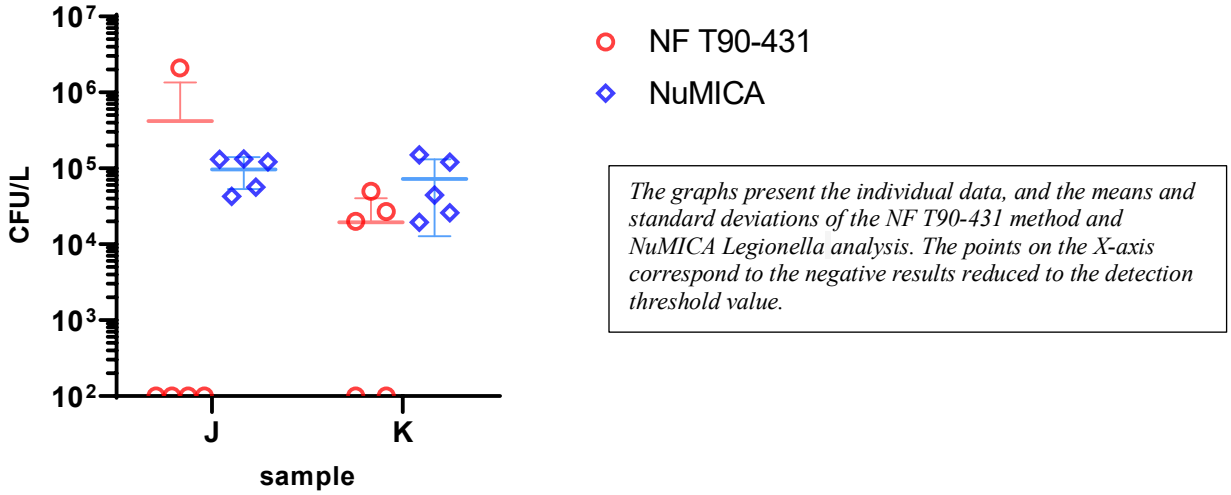
The study about cooling tower matrices was performed with two samples, J and K, naturally contaminated. Unlike the study on sanitary hot water matrices, the density of *Legionella pneumophila* in the sample was not known beforehand. Cooling tower water matrices generally contain a high density of interfering flora, which was indeed the case for the analyzed samples (Figure 4.2).



On the left, spread of 100 µL of sample J concentrated 100x (which corresponds to 10 mL of sample), after 3 days of culture at 37°C. On the right, 100 mL filtration membrane of sanitary hot water sample. The putative Legionella pneumophila colonies are small gray/cream bulging colonies. They must be isolated before being analyzed to confirm their identification.

Figure 4.2 Difference in interfering flora density between sanitary hot water and cooling tower water samples.

The analysis of cooling water samples by the NF T90-431 method gave very contrasting results: for sample J, one COFRAC laboratory gave a result greater than 1,000,000 CFU/L while the four other COFRAC laboratories gave a negative result; for sample K, three COFRAC laboratories gave a result between 10,000 and 100,000 while the two others gave a negative result. Conversely, the NuMICA *Legionella* solution produced much more homogeneous results (average standard deviation of 0.31 log units), without any negative results. From all these results, it can be deduced that *Legionella pneumophila* concentration was very high in these samples, but that NF T90-431 method gave a high rate of false negatives: 4/5 for sample J and 2 /5 for sample K (Figure 4.3 and Table 4.2).



The graphs present the individual data, and the means and standard deviations of the NF T90-431 method and NuMICA Legionella analysis. The points on the X-axis correspond to the negative results reduced to the detection threshold value.

Figure 4.3 Values obtained for cooling tower water with the two methods.

The very high proportion of false negatives from the NF T90-431 analyzes on these samples can be explained by the presence of large quantity of background flora. When counting colonies by eye on the agar media after 3 to 10 days of culture, the flora may have completely invaded the Petri dishes and covered up *Legionella pneumophila* colonies. Conversely, with NuMICA *Legionella*, the scan was made after 48 hours of growth only, when the background flora had not yet developed, allowing a better reliability of results, and limiting the false negatives risk.

Table 4.2 Summary of the results for cooling tower water.

	Sample	J	K		
NF T90-431	Mean [log10 (CFU/L)]	2,86	3,49		
	Std deviation	1,93	1,36	Mean of Std deviation	1,65
	False negative	4	2	Total false negative	6
NuMICA	Moyenne [log10 (UFC/L)]	4,94	4,72		
	Std deviation	0,23	0,40	Mean of Std deviation	0,31
	False negative	0	0	Total false negative	0

5. PRACTICAL IMPLICATIONS FOR RISK MANAGEMENT

Within the framework of "*Legionella* risk" control, the measures implemented in the different types of installations depend directly on the results of the analyses. In other words, the treatment actions are chosen according to the concentrations of microorganisms revealed by the detection methods, relatively to the thresholds to comply with. These thresholds differ from one country to another, and the main ones in France have been shown in Table 5.1 as an example.

Table 5.1 Thresholds and actions for « *Legionella* risk » management in France.

Installation type	Threshold (CFU/L)	Necessary action
Sanitary hot water, low risk institution	1000	Treatment
Sanitary hot water, high risk institution	10	Treatment
Cooling tower	1000	Treatment
	100 000	Shutdown of the incriminated circuit and treatment

When the same method produces variable results, as shown above, how to determine how close the considered result is to reality, and weather measures taken are relevant relatively to the real risk? Indeed, if the considered result is not within the correct concentration threshold, the action defined by the risk control processes will be inadequate: either ineffective (poor management of the health risk) or useless (application of treatment for no proven risk, therefore costly for the operator). In order to address this issue, a comparison of the two-by-two consistency of the results from the same method was carried out, based on the results shown in the paragraphs above. Each measurement was compared to each of the other measurements of the same sample. For sample A, 10 measurements were obtained (A1, A2… A10). A1 was compared to A2: A1 was within a defined interval; if A2 was within the same interval, the pair A1-A2 was scored as consistent; otherwise, the pair A1-A2 was noted as inconsistent (Figure 5.1). A1 was then compared to A3, A4 etc., up to A10. This method allowed to generate 45 pairs of results for each sanitary hot water sample, and 10 pairs for each cooling tower water sample.

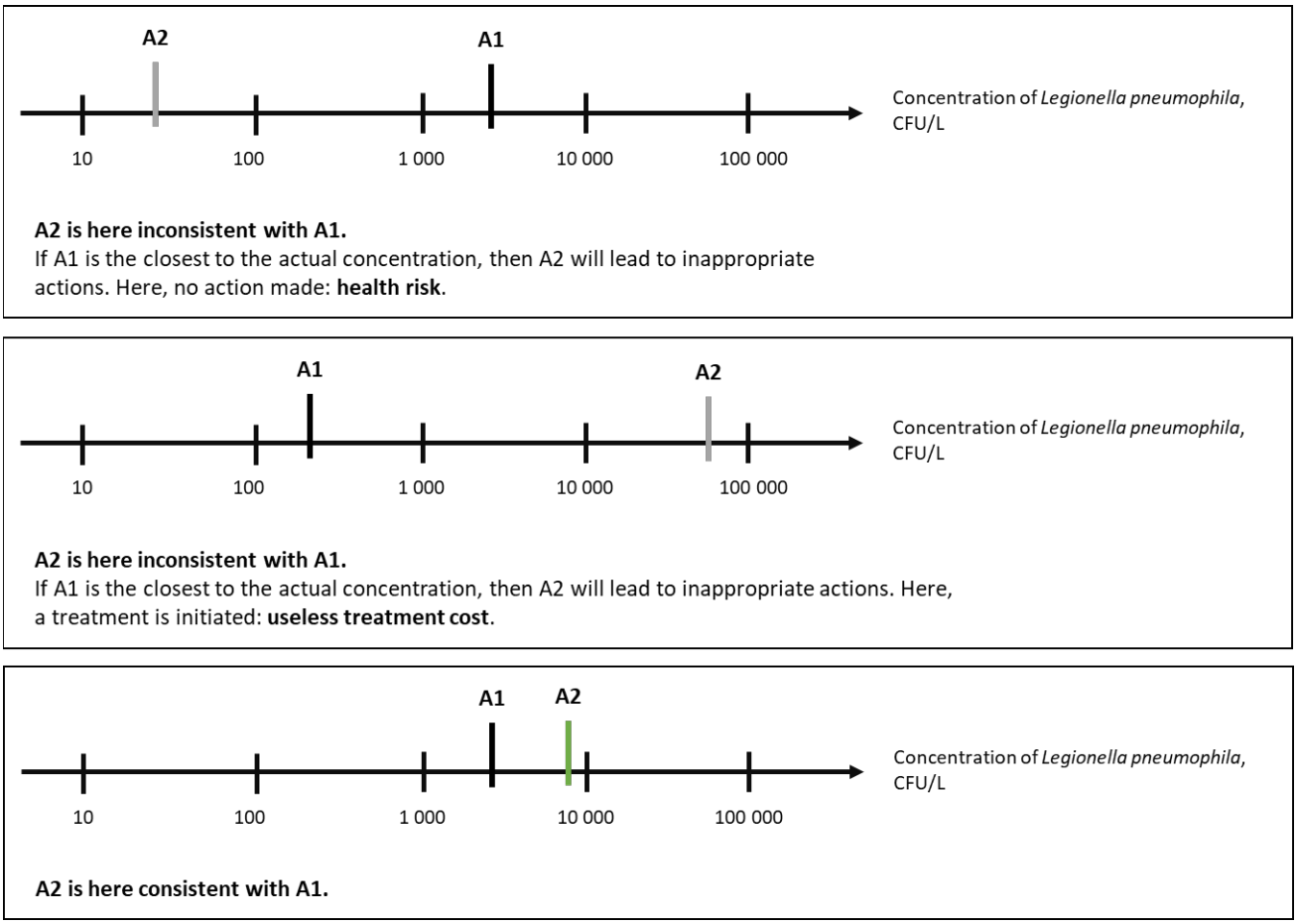


Figure 5.1 Classification principle of measurement pairs, relatively to action thresholds

The consistency (same interval) or inconsistency (different interval) score for each sample was subsequently determined by adding results of each comparison. For sample A, by using the NF T90-431 method, 13 pairs of measurements were consistent with each other, whereas 32 were not (Table 5.2). The same sample A tested with NuMICA *Legionella* showed 29 pairs of consistent measurements and 16 pairs of inconsistent measurements (Table 5.3).

Table 5.2 Classification of the comparisons between the different measurements of each sample by using the NF T90-431 method

	Pairs of consistent measurements	Pairs of inconsistent measurements
A	13	32
B	21	24
C	28	17
D	45	0
J	6	4
K	4	6
Total	58,5%	41,5%

Table 5.3 Classification of the comparisons between the different measurements of each sample by using NuMICA *Legionella*

	Pairs of consistent measurements	Pairs of inconsistent measurements
A	29	16
B	45	0
C	45	0
D	45	0
J	4	6
K	4	6
Total	86%	14%

Overall, with 11 samples tested, 58% from the NF T90-431 results were consistent with each other, which means that 42% of the results were likely to lead to incorrect actions and therefore probably to adverse consequences. With the same samples tested, 86% from NuMICA *Legionella* results were consistent with each other. Inadequate actions were therefore limited to 14%.

6. DISCUSSION

By using the NF T90-431 method for *Legionella* enumeration, operators must manage the high inherent variability related to (i) the volumes of sample deposited on the different agars, particularly when *Legionella pneumophila* concentration is low, (ii) the calculation method, (iii) human interpretation, and (iv) the potential interference with the flora present in the sample.

The NuMICA solution was created as a part of improving microorganism detection methods to minimize health and microbiological risks in water circuits. Indeed, the reduction in variability of results produced by NuMICA is reflected through the early detection of micro-colonies after only 48 h, therefore preventing interfering flora from developing importantly. In addition, NuMICA limits biases resulting from calculation method since a large number of micro-colonies are individualized on a reduced surface because the process of reading occurs when they have a small size, which makes it possible to count the entire density range on a single culture dish. Lastly, NuMICA removes bias related to human interpretation thanks to its automated features.

7. CONCLUSION

Due to increasing industrialization, cases of legionellosis continue to increase in many countries. Therefore, it becomes necessary to have control methods that are fast and reliable enough to monitor the evolution of these pathogens as accurately as possible. Since regulatory controls alone (when existing) are not sufficient for a good risk management, they must be supplemented by the implementation of regular self-monitoring. The choice of the method for this self-monitoring is decisive for the safety of workers and the durability of the installations.

The comparative assessment in this study highlighted the limitations of the conventional French method NF T90-431, analogous to the international method ISO 11731, for *Legionella* monitoring in water systems. Indeed, the NF T90-431 method presented significantly variable results, especially for samples whose matrices were loaded with background flora. This variability would clearly lead to overestimates or underestimates of the risks resulting in, on the one hand, unnecessary excessive actions with disadvantageous economic costs and, on the other hand, insufficient actions generating a high health risk which could lead to closure of facilities.

The NuMICA global solution was developed with the aim of drastically reducing inconsistent actions, by monitoring the quantitative evolution of pathogens such as *Legionella* in facilities. This evaluation also highlighted that NuMICA *Legionella* produced more reliable and faster results than conventional methods, offering operators the possibility of continuing their activities without any concerns.

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