Comparison of the Pseudalert™/Quanti-Tray® MPN test for the enumeration of *Pseudomonas aeruginosa* in cooling tower water with the ISO 16266 membrane filtration culture-based method

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Abstract

**Aims:** The purpose of this study was to evaluate the ISO 16266 membrane filtration culture-based method for the detection of *Pseudomonas aeruginosa* from cooling tower and related water samples and to compare the performance of the Pseudalert MPN method (Pseudalert™/Quanti-Tray®) for the enumeration of *P. aeruginosa* with the ISO method.

**Methods and Results:** Samples were analysed by both methods and the generated data were analysed according to ISO 17994 and showed that Pseudalert resulted in significantly higher counts and a better recovery of *P. aeruginosa* than the ISO method for 10 and 100 ml sample volumes.

**Conclusions:** Pseudalert represents a significant improvement in the enumeration of *P. aeruginosa* from cooling tower water and related samples. The advantages of Pseudalert became apparent in a better performance, a more than 10-fold higher upper quantification limit when using Quanti-Tray/2000 and a shorter incubation time with no requirement for further confirmation, resulting in a faster reporting of results.

**Significance and Impact of the Study:** The (Pseudalert/Quanti-Tray) MPN method offers a more efficient and sensitive test for enumerating *P. aeruginosa* from cooling tower waters, thus significantly improving management of occupational safety during cleaning and maintenance activities of cooling towers.

Introduction

Cooling towers dissipate heat from technical processes to the environment. They offer suitable conditions for microbial growth (Liu et al. 2011). The VDI Cooling Tower Code of Practice 2047-2 (Verein Deutscher Ingenieure, VDI 2015) is the German technical guideline for hygiene in cooling towers and recommends the detection of the biofilm-forming bacterium *Pseudomonas aeruginosa* in cooling tower waters. The biofilm-forming, Gram-negative bacterium *P. aeruginosa* is an opportunistic pathogen capable of causing bacteraemia, pneumonia, keratitis, otitis and infections of the soft tissue, the skin and the urinary tract (Gellatly and Hancock 2013). Bacteria in biofilms of industrial environments are resistant to biocides against biofouling processes (Costerton et al. 1995). The presence of *P. aeruginosa* in water systems is widely associated with biofilm development (Bédard et al. 2016) and, thus, it can be indicative of the presence of biofilm-associated pathogens such as *Legionella* sp. Furthermore, during cleaning and maintenance activities of cooling towers *P. aeruginosa* may cause illness (Wiatr 2002). In terms of occupational safety, it is necessary to monitor the *P. aeruginosa* contamination in cooling towers (VDI 2015). Furthermore, the bacterium can lead to corrosion problems by metabolizing nitrogen-based corrosion inhibitors (Wiatr 2002). Cooling tower operators are required to initiate disinfection sanctions above a concentration level of 100 CFU per 100 ml (VDI 2015). Using the standard membrane filtration-based culture method ISO 16266 (ISO 2006), the evidence of *P. aeruginosa* in cooling tower waters is complicated by the widespread...
contamination with other bacteria and their possible slime production. The Pseudalert™/Quanti-Tray® most probable number (MPN) test has been shown to give significantly higher results than the reference method for the detection of P. aeruginosa from hospital waters and statistically not significantly different results for swimming and spa pool waters (Sartory et al. 2015a, 2015b). Thus, Pseudalert provides a potential method for better recovery and faster assessment of the contamination of cooling towers with P. aeruginosa.

The ISO 16266 method is a membrane filtration procedure for samples with expected low bacterial contaminants in which a selected sample volume is filtered through a 0.45 μm pore size membrane filter, which is then placed on a selective cetrimide-containing agar (Pseudomonas CN agar) and then incubated at 36 ± 2°C for 40 ± 4 h. Blue-green pyocyanin-producing colonies are P. aeruginosa and do not need further confirmation, but presumptive colonies that are fluorescent but nonpyocyanin producing or are reddish or brown in colour require further testing to demonstrate the production of ammonium from acetamide using Nessler’s reagent. Cooling tower water often contains high numbers of interfering bacteria which can impede the counting of P. aeruginosa colonies resulting in an underestimation. Thus, ISO 16266 does not offer an ideal enumeration method for P. aeruginosa from this matrix. An alternative method for the enumeration of P. aeruginosa in cooling tower water is the Pseudalert MPN test. The powdered Pseudalert reagent utilizes a fluorogenic aminopeptidase substrate which is hydrolysed by P. aeruginosa leading to blue fluorescence under UV irradiation (365 nm). Pseudalert/Quanti-Tray is incubated at 38 ± 0.5°C for 24–28 h. Enumeration is achieved by reference to a table for the MPN for the number of positive wells in the Quanti-Tray. The method has a reported sensitivity of 94%, selectivity of 100%, false-positive rate of <1.0% and a false-negative rate of 6.5% (ISO 2018).

The aim of the study was to evaluate the ISO 16266 membrane filtration-based method for the enumeration of P. aeruginosa from cooling tower and related water samples and to compare the performance of the Pseudalert MPN method (Pseudalert/Quanti-Tray) for the enumeration of P. aeruginosa with the ISO method. In the first orienting part of this study 100, 10 and 1 ml test volumes were analysed by both methods to assess the applicability of each method and to determine the appropriate routine sample test volume. According to the data from this first part, the proposed routine test sample volume for the enumeration of P. aeruginosa with Pseudalert is 10 ml. In the second part of this study, test volumes of 10 ml were exclusively examined to evaluate the comparability of both methods.

Materials and methods

Routinely tested samples (n = 303) from industrial, naturally contaminated, cooling towers (n = 202) and related water samples from scrubbers (n = 26) and different process sites (n = 75) were analysed by ISO 16266 and by Pseudalert over a period from January to March 2016 and September 2017 to January 2018. Water was collected in sterile 250 ml polyethylene bottles containing sodium thiosulphate. All samples were routine samples of the Laboratory for Technical Hygiene at the Institute for Hygiene and Public Health of the University of Bonn. Due to the different parameters tested the residual volume in the bottle was sometimes not sufficient to perform all volumes with both methods. Samples were processed and analysed by both methods within 48 h after collection.

Study part 1

In the first part of the study, 100, 10 and 1 ml sample test volumes were analysed to evaluate the ISO method for the enumeration of P. aeruginosa in 107 cooling tower, 18 scrubber and 17 process water samples from 80 different sampling sites. These volumes were analysed as follows:

i 100 ml sample test volume: 100 ml samples were directly poured from the sample bottle each into two 120 ml vessels with antifoam up to the 100 ml mark.

ii 10 and 1 ml sample test volume: 20 or 2 ml of the original sample was transferred to 290 ml vessels and filled up to the 200 ml line with sterile deionized water. After agitating the diluted samples, each sample was divided by pouring 100 ml into two 120 ml vessels with antifoam up to the 100 ml mark.

Study part 2

In the second part of the study, only 10 ml volumes were analysed with both methods. Sample dilution was performed as described above. In this part of the study 95 cooling tower, 8 scrubber and 60 process water samples were tested from 64 different sampling sites, whereas some sampling sites with a reliable presence of P. aeruginosa were tested repeatedly.

Sample analysis

For ISO 16266 (ISO 2006) 100 ml samples or diluted aliquots were filtered through white nitrocellulose 0.45 μm pore size filters and transferred to Pseudomonas CN agar plates (Oxoid™; Thermo Fisher Diagnostics GmbH
Microbiology, Wesel, Germany) and incubated at 36 ± 2°C for 44 ± 4 h. Blue-green pyocyanin producing colonies were counted as P. aeruginosa. Presumptive atypical P. aeruginosa colonies that were fluorescent but non-pyocyanin producing or were reddish or brown in colour were tested for the production of ammonium from acetamide (Sifin Diagnostics, Berlin, Germany) with Nessler’s reagent (Sifin Diagnostics). Colonies producing ammonium were counted as P. aeruginosa. Colonies that were not able to produce ammonium were recorded as ‘nontargets’. Nonfluorescent colonies were recorded semiquantitatively as ‘nontargets’. According to ISO 8199 (ISO 2007) the range of the number of colony-forming units (CFU) grown on the filter should ideally be between 10 and 100 CFU but not exceed 200 CFU. Thus, for the evaluation of the ISO 16266 method, colony counts higher than 100 were recorded as ‘too numerous to count’ (TNTC). For the comparison of the culture method with Pseudalert ISO, counts up to 200 CFU were registered.

Pseudalert (IDEXX Laboratories, Westbrook, ME) is a commercially available testing system that consists of powdered reagent in blister pack format. The Pseudalert reagent was added to the 100 ml aliquoted samples and the dilutions. After dissolving and careful shaking, the samples were poured into the Quanti-Tray/2000 (IDEXX Laboratories) and immediately sealed in a Quanti-Tray Sealer Model Sealer PLUS using a Quanti-Tray/2000 rubber insert (IDEXX Laboratories). Sealed Quanti-Tray/2000 samples were incubated paper side down at 38 ± 0.5°C for 24–28 h. The trays were examined for the presence of blue fluorescence under UV light (365 nm). Positive and negative controls were performed according to the Pseudalert instructions.

Confirmation of isolate identity from Pseudalert positive wells

The specificity of Pseudalert was analysed by performing confirmations using Pseudomonas CN agar, which contains cetrimide as a selective agent. Confirmations of isolates from Part 1 of the study were performed at IDEXX Laboratories, Newmarket, UK. In Part 2 of the study confirmations on three positive wells from each Quanti-Tray/2000 were performed at the Laboratory for Technical Hygiene, Institute for Hygiene and Public Health, University of Bonn.

Confirmations of the positive wells were done by disinfection of the underside area of the well on the of the Quanti-Tray with an alcohol wipe and cutting a small opening in the membrane with a sterile scalpel. Using standard 4 mm loops, loopfuls of suspension from the wells were streaked onto Pseudomonas CN agar plates and incubated at 36 ± 2°C for 24 h after which the plates were examined for colonies of P. aeruginosa. Confirmation tests were conducted according to ISO 16266.

Data analysis

The data were analysed by McNemar’s test (McNemar 1947) to assess whether one method offered a higher recovery of P. aeruginosa. The $\chi^2$ values were calculated with the Edward’s correction for a small sample collective.

The comparative P. aeruginosa paired count data were analysed according to the mean relative difference approach of ISO 17994 (ISO 2014) to determine whether the two methods could be considered of equivalent performance or not. The mean relative difference was calculated by the mean of the relative differences ($x$) of each pair of counts using the equation $x = 100\left(\ln(a) - \ln(b)\right)$, where $\ln(a)$ is the natural logarithm of the count by the trial method (Pseudalert) and $\ln(b)$ is the natural logarithm of the count by the reference method (ISO 16266). Evaluation of equivalence is based on the mean relative difference and the expanded uncertainty ($W$) based on the standard deviation of the mean ($W = 2s/\sqrt{n}$), from which the lower ($X_L$) and higher ($X_U$) limits of the ‘confidence interval’ are calculated. Data with a zero count by one method had plus one added to each pair of counts prior to log-transformation. Data with counts that exceeded a method count limit by at least one method or had zero counts by both methods were excluded according to ISO 17994. MPN counts from Quanti-Tray/2000 were converted to nearest whole integers. The percentage value of the upper and lower limits was set at ±20% and ±20% as suggested by ISO 17994. The methods are considered of equal performance if the lower limit of the confidence interval is between −20% and zero and the upper limit is between zero and +20%. There is a significant difference if the mean relative difference and the confidence interval are above or below zero.

Data analyses were performed using Excel 2010/2013 (Microsoft Inc., Redmond, WA).

Results

Study part 1

The first part of this study was designed to evaluate the enumeration of P. aeruginosa (PA) from cooling tower and related water samples with ISO 16266 and to assess the appropriate sample volume for Pseudalert routinely investigated from cooling tower and related industrial water samples. One hundred and one 100 ml samples were analysed with the ISO method but, due to limited
volume of sample available, only 78 samples were tested with both methods. One hundred and forty 10 and 1 ml samples were tested with both methods.

Evaluation of ISO 16266 for the enumeration of *P. aeruginosa* in cooling tower and related water samples

To evaluate the ISO 16266 membrane filtration culture-based method the colony counts of *P. aeruginosa*, presumptive atypical *P. aeruginosa* colonies and other nontarget organisms were recorded for 101 of the 100 ml samples and for all 102 of the 10 and 1 ml volumes.

The presumptive atypical *P. aeruginosa* colonies were counted with respect to their reaction in the production of ammonium from acetamide with Nessler’s reagent either as PA (i.e. acetamide-positive) or nontarget organisms (i.e. acetamide-negative) (Table 1). For the 101 100 ml test volume samples, 105 presumptive atypical *P. aeruginosa* colonies were tested and only six colonies (5.7%) showed a positive reaction, 95 colonies (90.5%) showed a negative reaction and four colonies (3.8%) showed an ambiguous reaction. The colonies with ambiguous reactions were counted as nontarget organisms. Of the 111 tested colonies from 10 ml test volume samples, 12 colonies (10.8%) were confirmed as *P. aeruginosa* and 99 colonies (89.2%) were nontargets. Similar results were recorded for the 102 1 ml test volume samples.

The proportions of target (*P. aeruginosa*) and nontarget colony occurrence on Pseudomonas CN agar of ISO 16266 are shown. For the 78 100 ml sample analyses a valid count range (1–100 CFU) was recorded in 41 samples (53%), In 19 samples (25%) the *P. aeruginosa* concentrations were in the ideal range of 11–100 CFU. Five additional 100 ml samples were not countable because of sediment in the sample and/or very strong slime production of bacteria. From the 102 samples of 10 ml test volumes analysed with the ISO method, the results from 101 (one being not countable) (Table 2b) showed similar results to those for the 100 ml test volumes. The most frequently occurring colonies were nontargets from 88 samples (87.1%). In 46 samples of 10 ml test volume analyses (45.5%) the nontarget counts exceeded the upper quantification limit of 100 CFU. *Pseudomonas aeruginosa* was recorded in 41 samples (40.6%). In 19 samples (18.8%) the *P. aeruginosa* counts were in the ideal range.

In the 1 ml test volume samples (*n* = 101), *P. aeruginosa* was recorded in 29 samples (28.7%) (Table 2c). Only five counts (5.0%) were in the ideal counting range of 11–100 CFU range. Nontarget organisms were detected in 84 samples (83.2%).

In many cases the counting of *P. aeruginosa* was complicated by the presence of high numbers of nontarget colonies (including those presumptive *P. aeruginosa* colonies recorded as nonconfirming). Often merging colonies and blurred colony limits led to subjective counts.

**Comparative statistics of ISO 16266 and Pseudalert**

In Table 3 the distribution of the paired data and the McNemar’s test results of the different sample volumes are shown. For the 78 100 ml sample analyses a valid

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### Table 1: Presumptive atypical *Pseudomonas aeruginosa* (PA) colonies and their confirmation results

|                | 100 ml (n = 101) | 10 ml (n = 102) | 1 ml (n = 102) |
|----------------|-----------------|-----------------|----------------|
| Number of presumptive atypical PA colonies | 105             | 111             | 63             |
| Confirmed PA colonies                   | 95 (90.5%)      | 99 (89.2%)      | 57 (90.5%)     |
| Presumptive PA colonies confirmed as nontarget | 6 (5.7%)    | 12 (10.8%)      | 6 (9.5%)       |
| Ambiguous reaction                       | 4 (3.8%)        | 0               | 0              |

### Table 2: Proportionality of target (*Pseudomonas aeruginosa*) and nontarget colony occurrence on Pseudomonas CN agar of ISO 16266 for (a) the 100 ml sample volume, (b) the 10 ml sample volume and (c) the 1 ml sample volume

| P. aeruginosa count range | Number of samples with nontarget colonies within count range |
|---------------------------|-------------------------------------------------------------|
|                           | <1  | 1–10 | 11–100 | >100 |
| (a) 100 ml sample volume (n = 96) |     |        |        |      |
| <1                         | 14  | 3     | 2      | 33   |
| 1–10                       | 1   | 0     | 0      | 14   |
| 11–100                     | 0   | 1     | 16     | 1    |
| >100                       | 3   | 0     | 8      | 0    |
| (b) 10 ml sample volume (n = 101) |     |        |        |      |
| <1                         | 7   | 5     | 28     | 20   |
| 1–10                       | 1   | 0     | 5      | 13   |
| 11–100                     | 4   | 1     | 2      | 12   |
| >100                       | 1   | 0     | 1      | 1    |
| (c) 1 ml sample volume (n = 101) |     |        |        |      |
| <1                         | 15  | 23    | 26     | 8    |
| 1–10                       | 1   | 10    | 7      | 6    |
| 11–100                     | 1   | 0     | 3      | 1    |
| >100                       | 0   | 0     | 0      | 0    |
data set of 56 paired data was obtained (Table 3a). The other data exceeded the upper quantification limit (‘too numerous to count’, TNTC) with one or both methods. While 16 samples of the 78 total tested samples (20-5%) were TNTC with the culture method, only three were with Pseudalert (3-8%). Twelve of the ISO method TNTC samples were quantifiable (‘positive’) with the trial method (15-4%). In four samples (5-1%) the ISO-method the growth on the filter was not countable. In total, 20 samples were quantifiable with Pseudalert (25-6%) but were negative, TNTC or not countable with the ISO method, where only three samples were quantifiable with the ISO method but negative or TNTC with Pseudalert (3-8%).

For the 10 ml data set 137 out of 140 paired data were valid (Table 3b). The majority of the samples resulted in negative data pairs (53-3%). Forty samples were positive with both methods (29-2%). Two samples were TNTC and one sample was not countable with the culture method but positive with Pseudalert.

All 140 of the 1 ml test volume sample results were analysable, but 70-7% of the data pairs were negative (Table 3c). Fifteen samples were quantifiable with the ISO method, but negative with Pseudalert (10-7%).

Table 3  Distribution of data pairs and McNemar’s test results of positive/negative data pairs for (a) the 100 ml sample volume, (b) the 10 ml sample volume and (c) the 1 ml sample volume study part 1

| ISO 16266 | Pseudalert | Positive | Negative | Total |
|-----------|------------|----------|----------|-------|
| (a) 100 ml sample volume (McNemar’s test: $\chi^2 = 0.57, P = 0.45$) | | | | |
| Positive | 34 | 5 | 39 |
| Negative | 2 | 15 | 17 |
| Total | 36 | 20 | 56 |
| (b) 10 ml sample volume (McNemar’s test: $\chi^2 = 0.38, P = 0.54$) | | | | |
| Positive | 40 | 14 | 54 |
| Negative | 10 | 73 | 83 |
| Total | 50 | 87 | 137 |
| (c) 1 ml sample volume (McNemar’s test: $\chi^2 = 2.23, P = 0.14$) | | | | |
| Positive | 19 | 7 | 26 |
| Negative | 15 | 99 | 114 |
| Total | 34 | 106 | 140 |

whereas seven samples were quantifiable with Pseudalert, but negative with ISO 16266 (5-0%).

The outcomes of the McNemar’s tests indicate that the two methods are not significantly different for each sample volume (100 ml: $P = 0.45$; 10 ml: $P = 0.54$; 1 ml: $P = 0.14$).

The results of the mean relative difference approach according to ISO 17994 are shown in Table 4. For the 100 ml data set, 41 samples were included in the statistical analysis and the mean relative difference is 45-3%. The mean relative difference and the confidence interval limits were all above zero. Consequently, this indicates that the methods are different and Pseudalert offers a significantly higher recovery. The upper and lower limits of the confidence interval from the 10 ml data sets are both outside the ±20% criterion leading to an inconclusive outcome. For the 1 ml test volume data, the upper limit is above the +20% criterion and the lower limit is between zero and the lower −20% criterion. This also leading to an inconclusive outcome.

At the outset of the study, IDEXX recommended to test 10 ml sample volumes, because of concern that the antibiotics and reactions of Pseudalert might be inhibited by the cooling tower water matrix, and false-positive wells could emerge. In this study part, there was no statistical evidence that Pseudalert might be affected by the heterogenous constitution of industrial waters by taking 100 ml sample volume compared to 10 and 1 ml sample volumes. However, 100 ml sample volume tests were not continued as there was evidence that biocides and compounds to avoid scaling used in the treatment of cooling tower waters might influence the Pseudalert reactions in undiluted samples. More samples were necessary to obtain a statistically unambiguous result for 10 ml sample test volume. Further assessment of 1 ml samples was not continued because the action level for P. aeruginosa in cooling tower water is 100 CFU per 100 ml and each count would be a case of compliance.

Study part 2

In the second part of the study an additional 163 10 ml test volume samples were tested with both methods, from

Table 4  Mean relative difference analysis according to ISO 17994 for the data of study part 1 of the paired counts of Pseudomonas aeruginosa counts from 100, 10 and 1 ml sample volumes

| Sample volume | n | Mean relative difference | Standard deviation | W | $X_l$ | $X_u$ | Outcome |
|---------------|---|-------------------------|--------------------|---|-----|-----|--------|
| 100 ml        | 41 | 45-3%                   | 106-5%             | 33-3% | 12-0% | 78-6% | Methods different (Pseudalert higher) |
| 10 ml         | 64 | −12.9%                  | 141-7%             | 35-4% | −48-3% | 22-5% | Inconclusive |
| 1 ml          | 41 | −31.5%                  | 115-8%             | 36-2% | −67-6% | 4-7%  | Inconclusive |

n, number of observations; W, half width of the ‘confidence interval’ around the mean relative difference; $X_l$, relative difference at the lower ‘confidence limit’; $X_u$, relative difference at the upper ‘confidence limit’.
which 152 samples with valid data pairs were analysable by McNemar’s test.

Statistical analyses were performed firstly for the additional data only and then for the combined data sets of the two study parts. In Table 5, the distribution of the data pairs and the McNemar’s test results are shown. Of the 152 valid samples, 81 (53.3%) were positive with both methods and 47 samples (31.0%) were negative (Table 5a). Eighteen samples were negative with ISO but quantifiable with Pseudalert (11.8%), six samples were vice versa (3.9%). With the ISO method, seven samples exceeded the upper quantification limit that were quantifiable with Pseudalert (4.3% of 163 samples). In total, 106 samples were quantifiable with Pseudalert (65.0%) and 87 samples were positive with the ISO method (53.4%).

With respect to the combined data set shown in Table 5b (total \( n = 303 \), valid \( n = 289 \)), 121 samples were positive with both methods, 32 samples had counts with Pseudalert only (11.1% of the 289 valid samples) and 16 with ISO only (5.5%).

Table 5 Distribution of data pairs and McNemar’s test results of positive/negative data pairs for 10 ml sample volume of (a) the study part 2 and (b) the combined 10 ml data of both study parts

|                | ISO 16266 |        |        | Total |
|----------------|-----------|--------|--------|-------|
|                | Positive  | Negative |       |       |
| (a) 10 ml results of the second part of the study (McNemar’s test: \( \chi^2 = 5.04, P = 0.02 \)) | 81  | 18  | 99  |
| Positive       | 6  | 47  | 53  |
| Total          | 87 | 65  | 152 |
| (b) Combined 10 ml results of both study parts (McNemar’s test: \( \chi^2 = 4.69, P = 0.03 \)) | 121 | 32  | 153 |
| Positive       | 16 | 120 | 136 |
| Total          | 137| 152 | 289 |

The outcome of the mean relative difference analysis according to ISO 17994 (Table 6) is that the two methods are different (study part 2 10 ml: \( P = 0.02 \); combined 10 ml: \( P = 0.03 \)).

The appropriate data only and then for the combined data set shown in Table 5b (total \( n = 303 \), valid \( n = 289 \)), 121 samples were positive with both methods, 32 samples had counts with Pseudalert only (11.1% of the 289 valid samples) and 16 with ISO only (5.5%).

With Pseudalert 163 samples were quantifiable (52.6%) and with the ISO 137 samples (44.2%). In total, more samples were quantifiable with Pseudalert. For both data sets the McNemar’s test indicates that the methods are different (study part 2 10 ml: \( P = 0.02 \); combined 10 ml: \( P = 0.03 \)).

The outcome of the mean relative difference analysis according to ISO 17994 (Table 6) is that the two methods are different with Pseudalert showing significantly higher recovery of P. aeruginosa from cooling tower and similar water than the ISO 16266 method, as for both data sets the mean relative differences and their associated confidence intervals are above zero. The mean relative differences are 23.4% for the combined data set and 42.9% for the data from Study Part 2.

Confimation

Confirmation testing was conducted on isolates from 420 positive wells from Study Part 1 and from 344 wells from Study Part 2. The presence of P. aeruginosa was confirmed in 411 wells from study part 1 (97.9%) and in 335 wells from study part 2 (97.4%), in total leading to a false-positive rate of 2.4%. Occasionally, cocultures of P. aeruginosa and other bacteria were observed on the confirmation plates. Growth of other bacteria in the well did not seem to be important for the result interpretation.

Six samples from three different sampling sites led to false-positive signals. One cooling tower water from one sampling site was contaminated with oil that led to false-positive signals in four tested samples. The other false-positive results were obtained in one process water sample and in one scrubber sample.

Many samples had negative results with both methods indicating that negative wells of Pseudalert are truly negative. Therefore, negative wells were not tested separately.

Discussion

The Pseudalert/Quanti-Tray MPN method offers a suitable method for the enumeration of P. aeruginosa in cooling tower and related water samples. This method provides significantly higher results than the ISO method for 10 and 100 ml sample volumes. The appropriate sample volume for the enumeration of P. aeruginosa in cooling tower and related water samples is considered to be 10 ml. Cooling tower water is usually treated with biocides and compounds to avoid scaling and sometimes it is contaminated with substances of the production process. Without dilution of the sample these constituents might influence the Pseudalert reactions when analysing 100 ml sample volumes. In this study, one cooling tower sample contained oil substances that led to weak fluorescence and false-positive signals in Pseudalert. The bottle containing this sample showed fluorescence under UV light. It is a simple test to check if the sample is suitable for analysis with Pseudalert. In cases where contaminant fluorescence is detected the ISO method should be used.

Previous multi-laboratory comparative studies of Pseudalert and the reference membrane-filtration methods ISO 16266 or the UK The Microbiology of Drinking Water—Part 8 (MoDW Part 8, also using PACN agar) (SCA 2015) in hospital tap waters (Sartory et al. 2015a) and swimming and spa pool waters (Sartory et al. 2015b) tested routine water samples and artificially contaminated ones (because the number of positive samples from most of the participating laboratories tended to be very low). For routine hospital tap water samples, there were insufficient data for a conclusive statistical assessment, but the
Table 6  Mean relative difference analysis according to ISO 17994 for the data of study part 2 of the paired counts of Pseudomonas aeruginosa counts from 10 ml sample volume and for the combined 10 ml data set

|                | n  | Mean relative difference | Standard deviation | W       | Xₗ    | Xᵤ    | Outcome                  |
|----------------|----|--------------------------|--------------------|---------|-------|-------|--------------------------|
| Study part 2 data | 105| 42.9%                    | 106.7%             | 20.8%   | 22.0% | 63.7% | Methods different (Pseudalert higher) |
| Combined data   | 169| 23.4%                    | 124.5%             | 19.2%   | 4.3%  | 42.6% | Methods different (Pseudalert higher) |

n, number of observations; W, half width of the ‘confidence interval’ around the mean relative difference; Xₗ, relative difference at the lower ‘confidence limit’; Xᵤ, relative difference at the upper ‘confidence limit’.

data indicated an equal performance of both methods. For the artificially contaminated hospital tap water samples, Pseudalert provided higher counts (Sartory et al. 2015a). In the multilaboratory study of swimming and spa pool water samples both the routine and the artificially contaminated samples resulted in statistically inconclusive assessments but indicated potentially better performance of Pseudalert. Combined data of routine and artificially contaminated samples resulted in an ISO 16266 outcome that the two methods were not statistically significantly different (Sartory et al. 2015b). In both studies, interfering bacteria did not seem to have an influence as these water types usually have a low bacterial background. In this study, the interfering bacteria had a great impact on the ISO 16266 counts. Thus, the ISO method seems to be an inappropriate routine method for the enumeration of P. aeruginosa from cooling tower and related industrial water samples. The ISO method is suitable for water types with low bacterial contamination. Cooling tower waters are often highly contaminated. The ISO method is not able to suppress the growth of nontarget organisms adequately. P. aeruginosa was the only detected organism on membrane filters in only 6% of the 10 ml test volume samples examined. The predominant growth on Pseudomonas CN agar were nontarget organisms. The high number of bacterial contaminants might have led to underrepresented counts of the target organism P. aeruginosa. Sediment in the sample formed a layer on the filter membrane and complicated or even inhibited growth and counting of P. aeruginosa. Slime producing bacteria frequently caused merging colonies and blurred colony limits resulting in inhibited growth and in subjective counts of P. aeruginosa. Due to these facts the detection of P. aeruginosa in cooling water and related industrial water samples with the ISO method is subject to fluctuations which results in increased measurement uncertainty. The enumeration of P. aeruginosa with Pseudalert was not subject to such fluctuations and offered better measurement certainty in this study. According to ISO 8199 (ISO 2007) counts from membrane filters are reliable if the total number of colonies grown is between 1 and 200 and if the number of target colonies is between 10 and 100. In this study many cases occurred where more than 200 colonies were counted on Pseudomonas CN agar plates. Often the ideal counting range was exceeded. Furthermore, ISO 8199 proposes that it is preferable to use an MPN method if high bacteria contaminants are expected.

In previous studies, 99.3% of positive wells from hospital tap water samples were confirmed as P. aeruginosa with ISO 16266 and MODW Part 8 procedures (Sartory et al. 2015a) and 97.0% from 912 positive wells from swimming and spa pool water samples (Sartory et al. 2015b). In this study 97.6% of 746 tested positive wells were confirmed as P. aeruginosa. In most cases the false-positive rate of 2.4% is traced back to oil substances leading to weak signals under UV light. The high bacterial contamination and the chemical constitution of industrial water samples did not seem to have a general impact on the Pseudalert reactions in this study.

The advantages of Pseudalert for the enumeration of P. aeruginosa in cooling tower and related industrial water samples become apparent in a better performance, a more than 10-fold higher upper quantification limit using Quanti-Tray/2000, a shorter incubation time, the removal of the mercury-containing toxic Nessler’s reagent and the lack of confirmation steps resulting in a faster report of the results.

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Conflict of Interest

David Sartory was commissioned by IDEXX Laboratories to provide advice for the study, assist in the analysis of the data and in the writing of the manuscript.
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