Interreg North-West Europe Water Test Network

EUROPEAN UNION

European Regional Development Fund

End of Test Report
Diamidex MICA Legionella

Water Test Network

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Summary of Technology and Trial

Technology summary

DIAMIDEX developed and produced a new kind of analytical tool, called **MICA Legionella**. It allows the detection and quantification of the pathogenic bacterium *Legionella (L.) pneumophila* within 48h, which by the classical culture-based detection requires up to 10 days. Identification of *L. pneumophila* is done by metabolic lipopolysaccharide labelling which targets specifically the O-antigenic part of the bacterium. This new approach allows easy detection and enumeration of culturable *L. pneumophila* within 48h.
The labelling process relies on two phases: the *L. pneumophila*-specific metabolization of DIAMIDEX's patented molecule, a precursor legionaminic acid-N3, and its association with a fluorophore via bio-orthogonal click-chemistry reaction. The fluorescent signal emitted by the labelled bacteria is read after 48 hours of incubation, with MICA Fluorescence equipment, which automatically counts *L. pneumophila* microcolonies by MICA Legionella Artificial Intelligence able to discriminate signal from their background. The entire process is non-destructive for the bacteria. The TRL-level is assumed to 7/8, instruments are already existing and working.

Test plan summary

The objective of the trials is to evaluate the reliability and the robustness of MICA Legionella especially on natural samples of sanitary water.

Part 1: Preparation of the trials
The MICA instrument and equipment have to be delivered and installed at TZW. The TZW personal has to be trained. When everything is fixed so far, the tests with real samples can start.
TIME SCALE: About 1 week for installation and training.

Part 2: Measurements and tests with artificially contaminated water samples
Samples with strains of *Legionella pneumophila* (positive) and *Legionella anisa* (negative) in a wide concentration range (between 1 and 100.000 per 100 mL) are to be analysed with the ISO-method 11731 and MICA Legionella.
TIME SCALE: It is planned to analyse about 100 artificially contaminated samples, tests and analyses will need about 4 - 6 weeks.

Part 3: Test of MICA Legionella in parallel to ISO-method for about 6 months
40 - 60 natural sanitary water samples per month will be analysed with both technologies for 6 months.
TIME SCALE: It is planned to compare about 300 samples. As TZW receives about 50 samples per month, this will need about 6 months.

Part 4: Evaluation and data analysis
Can be done when all data are available. Some meetings between TZW and Diamidex might be necessary.
TIME SCALE: About 4 - 6 weeks.

Duration
The parts 1 and 2 were planned to be done in January/February 2022, depending when the equipment would be delivered to TZW. The Part 3 should start in March 2022 at latest, until August 2022.

Summary of the test

The letter of approval was sent out in January 2022 (10/01/2022). The instrument was brought to TZW in March 2022.

The TZW personal was trained by DIAMIDEX in the TZW laboratory end of March 2022, so the planned trial parts 2, 3 and 4 could be done in the time interval of end of March to November 2022.

Part 1: Preparation of the trials
The MICA instrument and equipment were delivered at TZW mid-March 2022. The TZW personal was trained and instructed in the use of the instrument.
TIME SCALE: Training by Sam Dukan with pre-prepared samples in 1 week in March 2022.

Part 2: Measurements and tests with artificially contaminated water samples
Samples with strains of *Legionella pneumophila* (positive) and *Legionella anisa* (negative) in a wide concentration range (between 1 and 100.000 per 100 mL) were analysed with the ISO-method 11731 and MICA Legionella.
TIME SCALE: This was done end of October 2022, after having done part 3 before. Because of time constraints, only 24 artificially contaminated samples could be analysed.

Part 3: Test of MICA Legionella in parallel to ISO-method for about 6 months
Natural sanitary water samples (hot drinking water sampled in buildings) were analysed with both technologies for about 6 months.
TIME SCALE: End of March until beginning of October 2022 TZW received a total of 215 samples for these analyses.

Part 4: Evaluation and data analysis
All data with both methods were to be compared and statistically evaluated. Some online-meetings between TZW and Diamidex were done.
TIME SCALE: This was done in November 2022.

Summary of results

The application for a voucher within the WTN-project was done by DIAMIDEX to evaluate the reliability and the robustness of MICA Legionella especially on natural samples of sanitary water.

The tests have been performed in TZW, which is a certified and accredited laboratory for the detection of *Legionella* spp. according ISO 11731. Moreover, TZW has a lot of experience with the identification of colonies by MALDI-TOF-MS, which had to be used additionally to be specific for *Legionella pneumophila*.

The tests in principle could be performed as planned except for some time constraints which lead to a somewhat lower number of samples than originally planned.

The results showed that the product MICA Legionella from the company DIAMIDEX works very reliable for the detection of *Legionella pneumophila*. In the natural sanitary samples analyzed at TZW within 7 months, there was a very good percentage of accordance between MICA Legionella and ISO-method 11731 (accordance of > 96%). Reaching such a good accordance after a short incubation time of 48 h is a very good progress and allows a good surveillance for health-relevant waters.

The instruments and the procedures are very robust and avoid human errors. The user guidance is very convenient for the laboratory personal.

An optimization of the algorithm was necessary to avoid underestimation of the concentrations when the membranes show a very dense growth of colonies. This was solved by Diamidex by changing the algorithm.

Some false negatives were observed for MICA Legionella compared to the ISO-method as used by TZW. This can be explained by the use of BCYE+AB-agar for the ISO-method, but GVPC-agar in MICA Legionella. BCYE+AB-agar is not as inhibitory as GVPC-agar, therefore a few more positive results in the low concentration range are possible. According the ISO-method, the use of both agars is allowed and in Germany BCYE+AB-agar is more often applied than GVPC-agar. In countries where only GVPC-agar is used also in the ISO-method, this low percentage of false negatives will not occur.

The results of these trials within Water Test Network led to some new insights for DIAMIDEX, but could show, that the system works as expected without problems. DIAMIDEX plans to bring MICA Legionella on the market as soon as possible.

Introduction

The patented MICA Legionella will be able to enumerate all culturable *L. pneumophila* in a water sample, from all serogroups, (i) in only 2 days (ii) with very low number of steps, (iii) with no human mistake, (iv) with very good accuracy and robustness and (v) at a very competitive price. In the new EC drinking water directive from December 2020, Legionella is included as a parameter for risk assessment in buildings.

The different European countries mostly use the regulatory approach to analyze Legionella with the cultural method described in ISO 11731. This detection method takes at least 10 to 14 days. So, the patented MICA Legionella decreases the detection time by a factor of 7. By using MICA Legionella it will be possible to better control this microbiological risk and reduce the number of exposed persons.

The MICA Legionella has already been under an evaluation test with three companies, EDF, NUVIA and AQUAPROX (a water treatment company), which could be potential customers. These are mainly water companies or end-users that manage cooling towers and facility manager companies that have to take care for hot-water systems in buildings.

The results are shown in Figure 1. Around 1000 different water samples coming from cooling tower or domestic hot water have been analyzed with both methods. The MICA Legionella solution reached very good sensitivity and specificity.

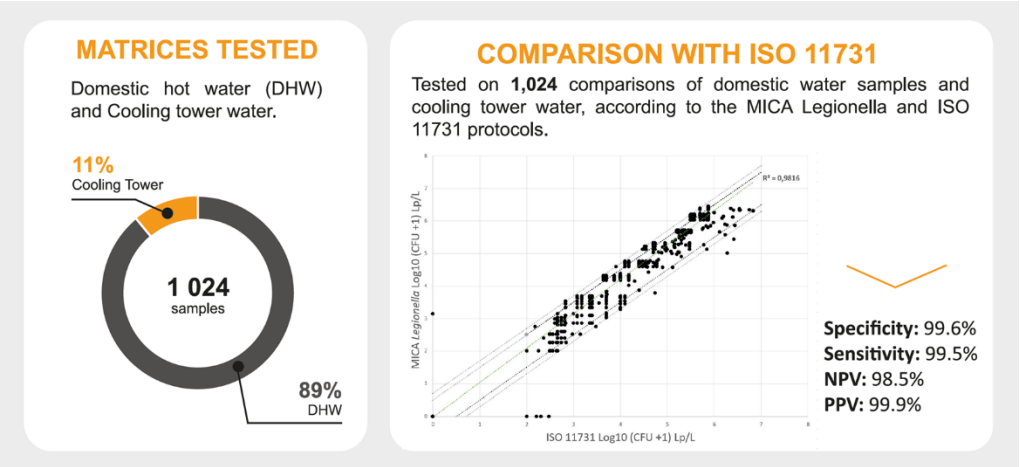


Figure 1 **Comparison tests for MICA Legionella so far**

DIAMIDEX made a stage 2-application on 07/01/2022 for the product MICA Legionella in the frame of the Water Test Network-project, which was accepted on 10/01/2022. The trial scope was estimated on total costs of € 41.600.

DIAMIDEX as technology provider was responsible to bring and install the MICA-instruments and equipment to TZW. Moreover, they had to do the first installation and the training, how the instruments were to be used and sampled. They should be available, if troubleshooting would be necessary. The produced data should be commonly evaluated.

TZW was included in the trial as **test-site provider** and as **analyzing laboratory**. Its responsibility included the analyses for *Legionella pneumophila* according the given protocol, as well as the standard analyses according the ISO-method. To make the ISO-method comparable to the MICA Legionella protocol, all positive *Legionella* spp. colonies had to be identified specifically by MALDI-TOF-MS.

The goal of the trials was to reach market readiness of the product.

Figure 2 shows the instruments as they were installed in the laboratory of TZW, including on the left hand side the filtration apparatus and on the right hand side the MICA Legionella reader.

Figure 2 Image of the MICA Legionella in the TZW laboratory.

Process

MICA Legionella is a new kind of analytical tool, which allows the detection and quantification of the pathogenic bacterium *Legionella (L.) pneumophila* within 48h. Identification of *L. pneumophila* is done by metabolic lipopolysaccharide labelling which targets specifically the O-antigenic part of the bacterium.

The labelling process relies on two phases: the *L. pneumophila*–specific metabolization of DIAMIDEX's patented molecule, a precursor legionaminic acid-N3, and its association with a fluorophore via bio-orthogonal click-chemistry reaction. The fluorescent signal emitted by the labelled bacteria is read after 48 hours of incubation, with MICA Fluorescence equipment, which automatically counts *L. pneumophila* microcolonies by MICA Legionella Artificial Intelligence able to discriminate signal from their background. The entire process is non-destructive for the bacteria.

In Figure 3, the procedure of the ISO-method for *Legionella* spp. is summarized, in Figure 4, the procedure of MICA Legionella.

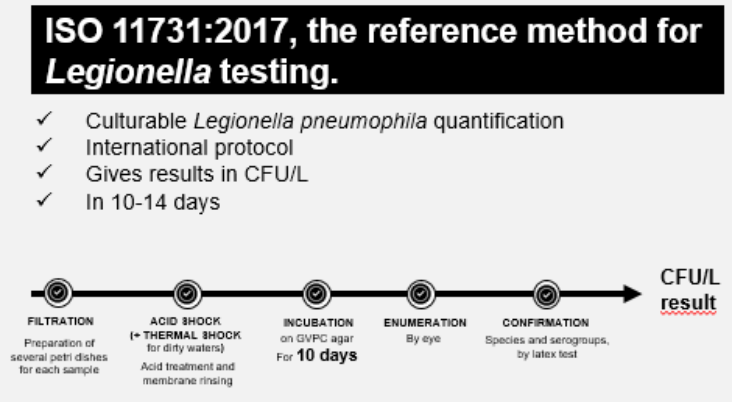


Figure 3 **Schematic procedure of the ISO-method 11731**

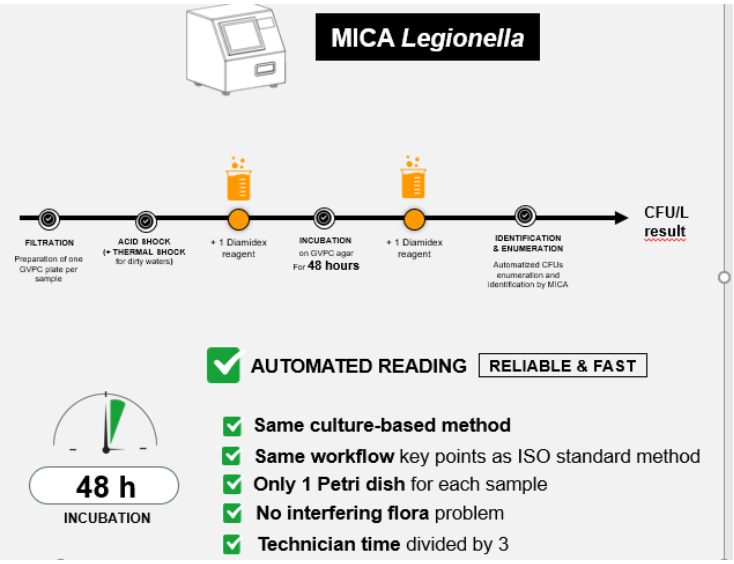


Figure 4 **Schematic procedure of the MICA Legionella-method**

Figure 5 shows the whole system, including washing bench, filtration apparatus and fluorescence reader. The entire process is guided by a user guidance tool which shows all actions in the given time intervals on the screen of the fluorescence reader. Some examples for the user guidance are shown in the Figures 6 and 7.

Figure 5 MICA Legionella system

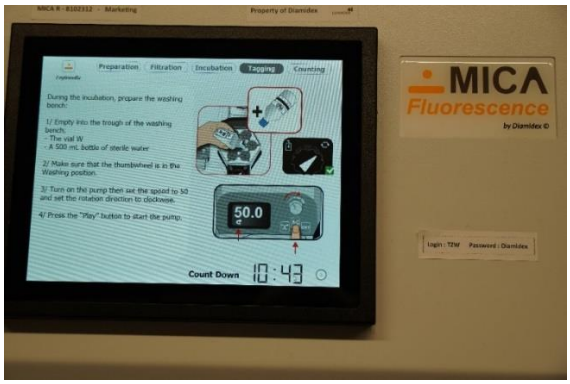


Figure 6 User guidance for tagging

Figure 7 User guidance for counting

The main works were to be done for the comparison tests in part 3 of the trials. Those were done with hot water samples from buildings. Those natural samples from sanitary water were routinely sampled and analyzed according ISO 11731 by accredited personal from TZW. The samples came mainly from hospitals or retirement homes.

Such samples have to be analyzed in Germany in public and private buildings according the German drinking water directive. There is a limit value given of 100 cfu *Legionella* spp. per 100 mL according ISO 11731.

So, these samples are normally only tested for the general detection of *Legionella spp*., not for a specific *Legionella pneumophila*-detection. Therefore, to be able to compare the ISO 11731-results with the MICA Legionella results, all positive *Legionella* colonies were additionally identified by a MALDI-TOF-MS-analyzer from Bruker.

Results

For DIAMIDEX, the application for a voucher within the WTN-project was done to evaluate the reliability and the robustness of MICA Legionella especially on natural samples of sanitary water.

The tests have been performed in TZW, which is a certified and accredited laboratory for the detection of *Legionella* spp. according ISO 11731. Moreover, TZW has a lot of experiences with the identification of colonies by MALDI-TOF-MS, which had to be used additionally to be specific for *Legionella pneumophila*.

Part 2 of the trials was done with artificially spiked water samples. All the results of this part are documented in the Annex I. The samples were spiked on one hand with *Legionella pneumophila* and on the other hand with *Legionella anisa*. The first ones should give positive results with MICA Legionella as well as with the ISO-method, the second ones should give negative results with MICA Legionella, but positive results with the ISO method for *Legionella* spp. Both series were analyzed in 6 different concentrations, each concentration in duplicate. So, 12 samples were analyzed for each of the two *Legionella* strains.

All *Legionella pneumophila* samples gave a positive response in the MICA Legionella and all the *Legionella anisa* samples gave a negative response in the MICA Legionella. The overview of the results is given in Table 1.

Table 1 **Results of MICA Legionella compared to ISO 11731 in artificially spiked water samples (on the left: numbers of analyses, on the right: percentages of analyses)**

n_{total}		24	MICA Legionella	
n_{total}		24	positive	negative
ISO-method	positive		12	0
	negative		0	12

n_{total}		100%	MICA Legionella	
n_{total}		100%	positive	negative
ISO-method	positive		50,0%	0,0%
	negative		0,0%	50,0%

So, the specificity was perfectly as expected. But the very high concentrations of *Legionella pneumophila* (8 from 12 samples) did give a number of *Legionella pneumophila* which was too low, so an underestimation for *Legionella pneumophila* (see Annex I). On these membrane filters, the number of colonies was too high for the used algorithm. This problem will be fixed with a new version of the algorithm, which indicates now for very densely grown membranes a value of "superior to 2 x 10^6 cfu / L".

Part 3 of the trial was done on a total of 215 natural samples from hot water systems in buildings. Those samples were analyzed with both methods. The results are summarized in Table 2. The main part of the sanitary samples (89 %) were giving negative results with both methods, about 6 % (13 samples) showed positive results with both methods, 2,8 % (6 samples) were negative with MICA Legionella, but positive with the ISO-method. Additionally, another 5 samples were also positive with the ISO-method and negative with MICA Legionella, but those 5 were very close to the detection

limit of the ISO-method (2 cfu / 100 mL, 20 cfu / L). In this range, the difference is not statistically significant, therefore those 5 samples have not been counted as “false negative”.

The reason for the somewhat less positives for MICA Legionella compared to the ISO-method might be the use of GVPC-agar with the MICA system, whereas with the daily ISO-method at TZW, BCYE+AB-agar is used. GVPC-agar contains more antibiotics and therefore is more inhibiting than BCYE+AB-agar. But anyway, the percentages of accordance are very good. They are reached already after 2 days of incubation with MICA Legionella.

Table 2 **Results of MICA Legionella in natural sanitary samples from TZW, compared to ISO 11731 in the time interval of April to October 2022**
(on the left: numbers of analyses, on the right: percentages of analyses)

n_{total}		215	MICA Legionella	
n_{total}		215	positive	negative
ISO-method	positive		13	6
	negative		0	191

n_{total}		100%	MICA Legionella	
n_{total}		100%	positive	negative
ISO-method	positive		6,0%	2,8%
	negative		0,0%	88,8%

The results from part 3 are additionally shown quantitatively in Figure 7. From the quantitative data it could be calculated, that the correlation of MICA Legionella with the ISO is 96,97% within 0,7 Log and 96,54% within 0,5 Log.

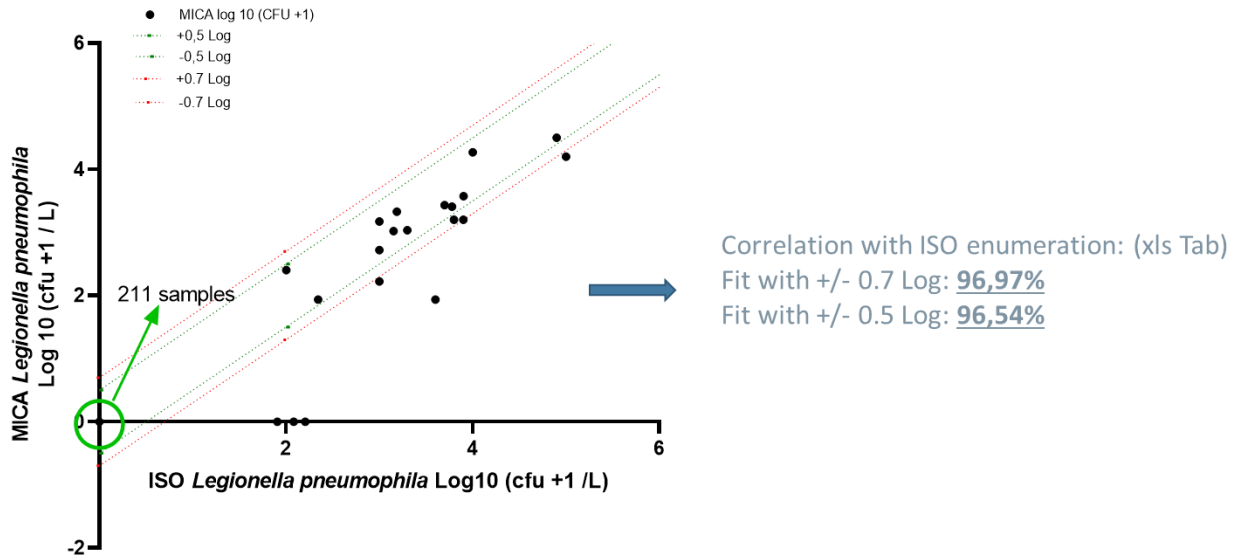
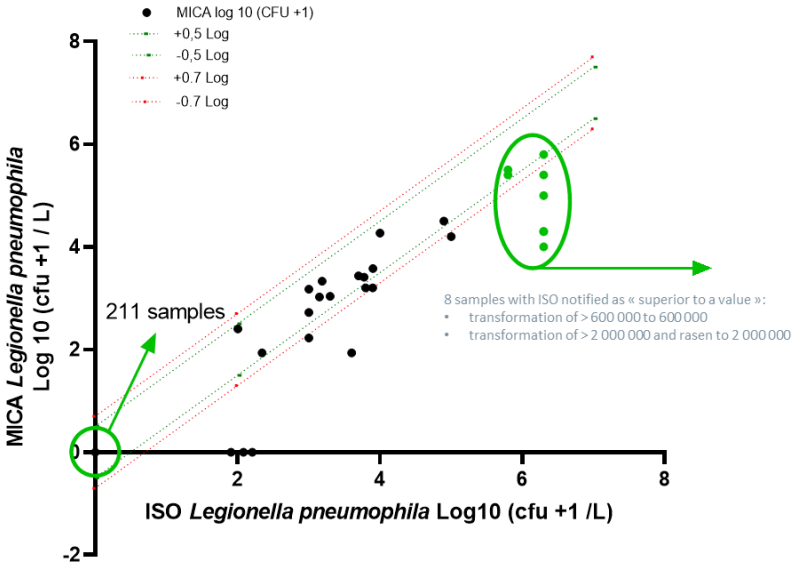


Figure 7 **Quantitative comparison of the natural sanitary samples analysed in TZW**

All the results including the spiked samples are shown quantitatively in Figure 8. For the spiked samples with high concentrations, higher deviations were seen, as the MICA Legionella numbers were too low. As a consequence, a new version of the algorithm of the instrument was created, which is now able to deal with this problem (see before).



spiked sample with very high density	MICA	ISO 11 731	MICA Log10	ISO Log10
pneumo 2	341551	>600000	5,53	5,8
pneumo 3	280215	>2000000	5,45	6,3
pneumo 4	10367	rasen	4,02	6,3
pneumo 5	20797	rasen	4,32	6,3
pneumo 2	243320	>600000	5,39	5,8
pneumo 3	582853	>2000000	5,77	6,3
pneumo 4	17947	rasen	4,25	6,3
pneumo 5	92728	rasen	4,97	6,3

Figure 8 **Quantitative comparison for natural sanitary and artificially spiked samples analysed in TZW**

Conclusions

The results showed that the product MICA Legionella from the company DIAMIDEX works very reliable for the detection of *Legionella pneumophila*. In the natural sanitary samples analyzed at TZW within 7 months, there was a very good percentage of accordance between MICA Legionella and ISO-method 11731 (accordance of > 96%). Reaching such a good accordance after a short incubation time of 48 h is a very good progress and allows a good surveillance for health-relevant waters.

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The results of these trials within Water Test Network led to some new insights for DIAMIDEX, but could show, that the system works as expected without problems. DIAMIDEX plans to bring MICA Legionella on the market as soon as possible.

Annex I

Part 2 Results of artificially spiked samples

WTN	DIAMIDEX MICA Legionella	Artificial samples		25.10.2022
Id_Sample	LIMS	Leg. pneumophila / L		Leg. spp. / 100 mL
MICA ID	TZW ID	MICA	ISO + MALDI	ISO
88	Legionella pneumophila 0 a	1566	8000	800
88	Legionella pneumophila 1 a	28293	78000	7800
88	Legionella pneumophila 2 a	341551	>600000	>60000
88	Legionella pneumophila 3 a	280215	>2000000	>200000
88	Legionella pneumophila 4 a	10367	overgrown	overgrown
88	Legionella pneumophila 5 a	20797	overgrown	overgrown
89	Legionella pneumophila 0 b	1496	7000	700
89	Legionella pneumophila 1 b	15336	93000	9300
89	Legionella pneumophila 2 b	243320	>600000	>60000
89	Legionella pneumophila 3 b	582853	>2000000	>200000
89	Legionella pneumophila 4 b	17947	overgrown	overgrown
89	Legionella pneumophila 5 b	92728	overgrown	overgrown
90	Legionella anisa 0 a	<= 40	<20	400
90	Legionella anisa 1 a	<= 40	<20	2200
90	Legionella anisa 2 a	<= 40	<20	13500
90	Legionella anisa 3 a	<= 40	<20	>60000
90	Legionella anisa 4 a	<= 40	<20	>200000
90	Legionella anisa 5 a	<= 40	<20	overgrown
91	Legionella anisa 0 b	<= 40	<20	400
91	Legionella anisa 1 b	<= 40	<20	2500
91	Legionella anisa 2 b	<= 40	<20	19800
91	Legionella anisa 3 b	<= 40	<20	>60000
91	Legionella anisa 4 b	<= 40	<20	>200000
91	Legionella anisa 5 b	<= 40	<20	overgrown