

**Report of results
concerning evidence of efficacy of chosen biocides
in water soluble metalworking fluids against
mycobacteria – especially *M. immunogenum***

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Initial situation

Since the early 1990s, repeated cases of respiratory diseases in the form of a hypersensitivity pneumonitis (HP) occurred in the USA at several automotive manufacturers and suppliers. In used coolant samples of water soluble metalworking fluids (MWF), a Mycobacterium (M) – *M. immunogenum* –, which until then was not known as an independent species, could be isolated. An insufficient efficacy of certain N-formals, especially substituted hexahydrotriazines (sHHT) against this species of bacteria (1) is assumed. For this reason, a large automotive corporate group strictly limited the use of N-formals in water soluble metalworking fluids at that time.

After a similar situation occurred at an automotive manufacturer in England in 2003 (2) without *M. immunogenum* being able to be found in used coolant samples, forty samples from metalworking fluid companies were tested for the presence of mycobacteria in a test series initiated by the Berufsgenossenschaft Metall Nord Süd (BGM, former Süddeutsche Metall-Berufsgenossenschaft, Mainz). *M. immunogenum* could only be found in one sample originating from the USA (3). Due to these results, a hazard caused by mycobacteria for activities with water soluble metalworking fluids was not assumed in Germany.

In the beginning of 2007, *M. immunogenum* was for the first time found in a remarkably high concentration in a metalworking fluid sample preserved with N-formals taken in a central plant within the framework of a procedure for the identification of occupational diseases of the BGM. Although in the meantime several companies are concerned, only in the first case an accumulation of respiratory diseases occurred, which were recognized as occupationally caused HP.

In all other companies where *M. immunogenum* was identified in water soluble metalworking fluids, no diseases could be observed in this context until now.

Comparing studies of a biocide supplier in Germany in 2006 on the efficacy of different biocides confirmed the insufficient efficacy of triazines and oxazolidines (N-formals) especially against the species *M. immunogenum*, which had already been postulated in the USA. The internal study was done in cooperation with the "Nationales Referenzzentrum (NRZ) für Mykobakterien, Borstel".

The above investigation approach of a comparing biocide efficacy against mycobacteria was taken up by the BGM and extended in two test series. The strains of *M. immunogenum* and of another type of mycobacteria not yet described, which had been isolated by then from used coolant samples of metalworking fluids in Germany, were used. This is of importance insofar as the strain cultures (ATCC strains) usually used in test series may reveal a different behavior than the „wild strains“ from native samples.

Two types of mycobacteria isolated from patient material served as reference strains; in addition, another metalworking fluid containing derivatives of boric acid was used.

Material und methods

Bacteria strains:

Mycobacterium immunogenum (strain Nos. 925/07 and 1388/08).

Mycobacterium of an unknown type (strain No. 1385/08)

Mycobacterium gordonae (strain No. 719/08).

→ the above strains were isolated from different used coolant samples of water soluble metalworking fluids at the NRZ.

Mycobacterium avium (strain No. 3392/08)

Mycobacterium fortuitum (strain No. 3838/08)

→ the above strains were isolated from patient material at the NRZ

Metalworking fluids:

MWF A	Containing mineral oil (30%) Water soluble, free from derivatives of boric acid, containing amine Typical concentration of use: 3-15% pH value at 3% = 9,2
MWF B	Containing mineral oil (75%) Water soluble, free from derivatives of boric acid, containing amine Typical concentration of use: 5-10% pH value at 5% = 9,1
MWF C	Containing mineral oil (40%) Water soluble, containing derivatives of boric acid (about 8%), containing amine Typical concentration of use: 4-6% pH value at 4% = 9,0

The metalworking fluids were provided for the planned investigation purposes without being preserved in advance and were used in the test series in concentrations of 2,5% and 5%.

Biocides:

Abbreviation	Substance	Conc. of use in the test	Substance group
MBO	3,3'-Methylenbis[5-methyloxazolidine]	0,1%	N-formal
HHT	2,2',2''-(Hexahydro-1,3,5-triazine-1,3,5-triyl)-triethanol	0,1% und 0,15%	N-formal
EDDM	(Ethylendioxy)-dimethanol	0,1%	O-formal
CMIT/MIT	5-Chloro-2-methyl-2H-isothiazol-3-on + 2-Methyl-2H-isothiazol-3-on (relation 3:1)	0,1%	Isothiazolinones
EDDM + CMIT/MIT		0,1%	Formulation from O-formal + Isothiazolinone

Execution

40 ml of a 2,5 and a 5% emulsion were produced by mixing metalworking fluid and sterile WSH (water of standardised hardness = 17,0 dH (German hardness)). Following complete homogenisation, these emulsions were inoculated with the different bacterial strains, the initial bacterial count being about 10^6 CFU/ml. The inoculated emulsions were then spiked with the five biocides in a concentration of 0,1% resp. 0,15%. As the quantity of active ingredient in the HHT is lower than in the MBO and EDDM, the concentration of HHT was increased to 0,15% in the second test series. Due to the large inspection scope, this approach could only be done with the product containing derivatives of boric acid.

After 3, 8 and 23 days of residence time of the biocides at 25°C, the initial suspensions were subsequently inoculated with Löwenstein-Jensen medium in test tubes and with Middlebrook medium on plates. In order to stop the biocide action prior to incubation, the mixture from metalworking fluid, biocide and bacteria was diluted 1:100 with distilled water following the respective residence time.

The time of incubation was 3 weeks for the quickly growing mycobacteria and 8 weeks for the slowly growing ones.

Results

The following tables of results (Tables 1-3) combine two test series. A first test series partially differed as to test approach, evaluation and inspection scope as follows:

- only 1 *M. immunogenum* strain (Nr. 925/07) was used,
- the concentration of use of HHT was at 0,1%,
- the residence time of the biocides was 4, 11 and 16 days.

These differences are noted in the table or taken into account in the evaluation.

Key:

	++	= confluent growth (thick bacteria bed)
	+	= little growth
	+/-	= 1-5 single colonies
	-	= no growth

As the evaluation comprises three different residence times of the biocides used with possibly up to three different results, an additional coloured presentation using the „traffic light system“ was additionally chosen for simplification with the following conventions:

- 1.) The results for „little growth“ (+) and „1-5 single colonies“ (+/-) are represented by one colour.
- 2.) The result most frequently found determines the assignment to a colour.
- 3.) In case of a progression (e.g. no growth - little growth - bacteria bed), the assignment is done according to the middle result.

Table 1

MWF conc.	<i>M. immunogenum</i> (MWF strain 1)		<i>M. immunogenum</i> (MWF strain 2)		unknown mycobacterium (MWF strain)		<i>M. gordonae</i> (MWF strain)		<i>M. avium</i> (patient material)		<i>M. fortuitum</i> (patient material)	
	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%
MWF A (containing mineral oil (30%), free from derivatives of boric acid, containing amine)												
Control	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ ++	+/- +/- +/-	+/- - -	+ + +	+ + +	++ ++ ++	++ ++ ++	++ + +	- - -
HHT * (0,1%) 1. test series	++ ++ ++	++ ++ ++										
MBO (0,1%) 1. test series	++ - -	++ ++ ++										
EDDM (0,1%)	+ + +	+ + +	+ + +	+ + +	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
CMIT/MIT (0,1%)	+ + +	- + +	- - +/-	- - +/-	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
EDDM + CMIT/MIT (0,1%) 1. test series	- - -	- - -										

* HHT = 2,2',2''-(Hexahydro-1,3,5-triazine-1,3,5-triyl)-triethanol (CAS Nr. 4719-04-4)

Table 2

MWF conc.	<i>M. immunogenum</i> (MWF strain 1)		<i>M. immunogenum</i> (MWF strain 2)		Unknown mycobacterium (MWF strain)		<i>M. gordonae</i> (MWF strain)		<i>M. avium</i> (patient material)		<i>M. fortuitum</i> (patient material)	
	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%
MWF B (containing mineral oil (75%), free from derivatives of boric acid, containing amine)												
Control	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ ++	+/- +/- -	+/- - -	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ ++	++ ++ ++
HHT * (0,1% !) 1. test series	++ ++ ++	++ ++ ++										
MBO (0,1%) 1. test series	++ ++ +	++ + -										
EDDM (0,1%)	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
CMIT/MIT (0,1%)	+/- - -	- - -	- - +/-	- - -	- - -	- - -	++ - -	++ - -	++ ++ -	++ + -	- - -	+ - -
EDDM + CMIT/MIT (0,1%) 1. test series	- -	- -										

* HHT = 2,2',2''-(Hexahydro-1,3,5-triazine-1,3,5-triyl)-triethanol (CAS Nr. 4719-04-4)

Table 3

MWF conc.	<i>M. immunogenum</i> (MWF strain 1)		<i>M. immunogenum</i> (MWF strain 2)		Unknown mycobacterium (from MWF)		<i>M. gordonae</i> (from MWF)		<i>M. avium</i> (patient material)		<i>M. fortuitum</i> (patient material)	
	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%	2,5%	5,0%
MWF C (containing mineral oil (40%), <u>containing derivatives of boric acid acid (8%), containing amine</u>)												
Control	- + +	+ - -	+ + +	- + +	- - -	- - -	- - -	+/- +/- +/-	++ ++ ++	++ ++ ++	- - -	+/- - -
HHT * (0,15% !)	+/- +/- +/-	+/- - -	+/- + +	- + +	- - +/-	- - -	- - -	- - -	- - -	- - -	+/- - -	- - -
MBO (0,1%)	+ + +	+/- +/- +/-	+/- + +	+/- + +	+/- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
EDDM (0,1%)	- +/- +	+/- - -	+/- + +	+/- + +	- +/- -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
CMIT/MIT (0,1%)	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	++ - -	++ - -	- - -	- - -
EDDM + CMIT/MIT (0,1%)	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -

* HHT = 2,2',2''-(Hexahydro-1,3,5-triazine-1,3,5-triyl)-triethanol (CAS Nr. 4719-04-4)

Discussion

In the previous investigations of a biocide supplier on biocide efficacy, it already became obvious that *M. immunogenum* showed a different behaviour towards the biocides used in the test than the reference types *M. terrae* and *M. avium*. While HHT and MBO in the mentioned investigations were found to be insufficiently effective for *M. immunogenum*, the formulation CMIT/MIT lead to a very good result, the reverse was true for the tested reference strains.

The test series of the BGM again showed a restricted to insufficient efficacy of both N-formals. In contrast to the investigation mentioned above, this time, the formulation CMIT/MIT did also reveal insufficient efficacy, at least in the first MWF product. For EDDM as single active substance, the results differed with the metalworking fluid product used. The efficacy of the biocides is obviously influenced by the MWF product used.

As shown by the test approaches with the controls, all of the metalworking fluids, which are not preserved in advance for test purposes, offer rather good growth conditions for mycobacteria. Only the product containing derivatives of boric acid showed more stability against a colonisation with mycobacteria already in the biocide-free version. The N-formals and the O-formal tested with the metalworking fluid product containing derivatives of boric acid did not cause a recognisable improvement of the better microbiological stability already evident in the control. Thus, it is not possible to say, if the higher HHT concentration of 0,15% used in the second test approach leads to a stronger biocide effect. Thus, the question remains, if the efficacy of HHT resp. N-formals especially against mycobacteria is principally insufficient or if it is just the concentration used which is too low?

In the fluid samples containing *M. immunogenum* from the member companies of the BGM available up to now, a rather high total colony number of ubiquitous bacteria became obvious, which as well might indicate insufficient biocide efficacy or insufficiently high dosing. It is noteworthy that *M. immunogenum* was not found in any water sample (mixing water) while on the other hand, the mycobacteria typically existing in water like *M. xenopi*, *M. goodnae*, were not found in metalworking fluid samples with few exceptions. If *M. immunogenum* could be found in used coolant samples from the metalworking industry, it generally was the only mycobacterium and the concentrations were high (positive primary preparation = >10.000 CFU/ml).

The „unknown“ type of mycobacteria isolated from MWF samples is very sensitive towards the biocides used and shows a behaviour comparable to the other reference types.

In the test series carried out up to now, the preparation EDDM + CMIT/MIT resulted in reliable and reproducible biocide efficacy against all mycobacteria tested, including *M. immunogenum*. The biocide effect already occurs after three days of exposure. It has, however, to be noted that even after successful preservation *M. immunogenum* could still be shown in the “biofilm” (sessile bacteria) inside the plants, though eliminated from the fluid (in the form of planctonic bacteria). If the “biofilm” is not removed, a subsequent re-contamination of the metalworking fluid has to be assumed.

At present it is not possible to assess, if the cases of illness (HP) which occurred in connection with the evidence of *M. immunogenum* are solely a singular company-related event or if the possibility of more frequent occurrence has to be anticipated in the future. According to the current state of knowledge, *M. immunogenum* has to be classified as a suspected allergen for the elicitation of an HP, a definite etiological allergenic component could not be verified until now. Furthermore, it cannot be excluded that the course of events is multi-factorial and that presently unknown co-factors play a decisive role.

Summary

- 1.) In conventional concentrations of use, HHT and MBO (N-formals) do not prove sufficient efficacy against *M. immunogenum* strains isolated from the MWF samples.
- 2.) Compared to other types of mycobacteria, *M. immunogenum* shows higher stability to most of the biocides tested.
- 3.) The efficacy of EDDM (O-formal) and CMIT/MIT (N,S-heterocycles) as single formulation is indifferent and varies according to MWF product used resp. species of mycobacteria.
- 4.) The composition of the MWF product influences the efficacy of the biocides used.
- 5.) The MWF product containing derivatives of boric acid shows a good stability to colonisation with mycobacteria already in the non-preserved state (formulation without biocides) and thus also the best effect of the biocides tested.
- 6.) The formulation EDDM + CMIT/MIT proved a quick-acting and sufficient efficacy against all types of mycobacteria used in the test, including *M. immunogenum*.

Literature

- 1.) „White Paper – Hypersensitivity Pneumonitis: Is There an Association with Triazine Biocides and Mycobacteria in Metalworking Fluids?“, Safety, Health, Environmental Regulatory Affairs Committee of the Independent Lubricant Manufacturers Association (ILMA), USA
- 2.) Outbreak of respiratory disease at Powertrain Ltd, Longbridge, Birmingham Health and Safety Executive (HSE), 2006, England
- 3.) Report of results concerning the incidence of Mycobacteria (esp. *M. immunogenum*) in water-mixed metall working fluids (MWF) from industrie, April 2004, (<http://www.bg-metall.de> → Prävention → Biologische Noxen → Kühlschmierstoffe)