Microbial Arrays and Pattern Recognition for analysis of toxicity of chemicals

Inger Kühn*, Jenny Gabrielson, Ruth de Karzow, Patricia Colque, Roland Möllby

Microbiology and Tumorbiology Center, Karolinska Institutet, Box 270, 171 77 Stockholm, Sweden

Running title: Toxic fingerprints of chemicals

Abbreviations:

MARA	Microbial Assay for Risk Assessment
MTC	Microbial Toxic Concentration
LC50	50 % lethal concentration
IC50	50 % inhibitory concentration
MEIC	Multicentre Evaluation of In Vitro Cytotoxicity list

* Corresponding author. Phone +46 8 5248 7155; Fax +46 8 5248 7908; E-mail inger.kuhn@mtc.ki.se

Abstract

A toxicity test based upon a standardised array of eleven bacterial strains has previously been developed (named MARA). The bacteria are exposed to a gradient of the compound to be tested. The results are expressed as mean toxic concentrations of the chemicals towards the bacteria, as well as "toxic fingerprints" of the chemicals, that can be subjected to cluster analysis. In the present study, MARA was evaluated by testing a set of organic chemicals with similar chemical structure (the chlorophenols), a set of chemicals with different structure, but used for similar purposes (disinfectants), as well as a set of inorganic chemicals (metal salts). The toxicity of the tested chlorophenols in MARA agreed well with findings for other organisms, and cluster analysis of the toxic fingerprints could yield further information on similarities in the mode of action. For some of the disinfectants there was a large span in the concentrations needed to inhibit growth of different bacteria, whereas individual metal salts often showed similar toxicities for all tested bacteria. It was concluded that MARA could be useful test for dereplication of new chemicals that are produced e.g. in the pharmaceutical industry, as well as for evaluation of the toxicity of old and new chemicals, in order to get an idea about their biological properties and modes of action.

Keywords: microorganisms, toxicity testing, chlorophenols, disinfectants, metals, cluster analysis

Introduction

There is a need for alternatives to animal experiments to test chemicals and pollutants with regard to their effects on humans, animals and ecosystems. Alternative methods for toxicity testing are often based on mammalian cell cultures (e.g. human), or lower organisms, such as *Daphnia* (OECD guidelines) and sea urchin larvae (12). The latter are particularly suited for studies of ecotoxicity, where the concern is for the effects on organisms other than humans. Micro-organisms, particularly bacteria, are very suitable test organisms, for several reasons. They can easily be cultivated in large quantities, and can be freeze dried and stored for very long periods of time, which makes them particularly suitable for the production of standardised kits that can be made commercially available. They are genetically and biochemically very diverse - it is estimated that bacteria alone are responsible for more than 60% of the total genetic diversity on earth (17), and this offers the potential to set up an assay containing a combination of different microbial strains representing different taxonomic groups (5). They also have short generation times and may thus show responses to added chemicals rapidly.

Some bacteria-based test systems are widely used, such as the commercially available Microtox ™ and ToxAlert™ (9), which are assays based on the bioluminescent bacterium *Vibrio fisherii* (previously known as *Photobacterium phosphoreum*). Other assays based on natural or genetically constructed bioluminescent bacteria have also been described, e.g. (7, 10). A drawback of most such assays is that they yield information on the toxic activity on one organism only.

We have previously reported on the development of MARA (Microbial Assay for Risk Assessment) (4). MARA constitutes a toxicity test based upon a standardised array consisting of several (at least eleven) bacterial strains, belonging to a diverse range of taxonomical groups that are exposed to a gradient of the compound of which the toxicity is to be tested. The test is performed in microplates containing twelve columns and eight rows, thus allowing the simultaneous testing of eleven bacteria (one column is control without bacteria) with seven different concentrations (one row is negative control) of the chemical to be tested (Fig. 1). The microplates are read with a flat bed scanner, and especially developed software determines the toxic activity and the toxic "fingerprint" of the tested compound (3). The toxic fingerprints from different chemicals can be compared, and thus the assay, besides of yielding a

simple estimate of the level of toxicity of the compound towards micro-organisms, also may yield information on similarities in toxic activities between different chemicals, that also could reflect similarities in their mechanisms of action (4).
In the present study we have further evaluated the concept of toxic fingerprinting with MARA by studying the responses of an array of eleven bacterial strains to some families of chemical compounds with different properties. These were the chlorophenols - a group of chemicals with similar chemical structure, which are often used as model compounds in toxicity testing, disinfectants, which are chemicals with varying composition, but used for the same purpose, namely to kill bacteria, and metal salts.

Materials and methods

Bacterial strains

Eleven bacterial strains, mainly belonging to different genera, were used (Table 1). Ten of them were obtained from the National Collections of Industrial Food and Marine Bacteria (NCIMB, www.ncimb.co.uk), and had previously been subject to species identification with 16S DNA sequencing. The *Enterococcus* strain was obtained from the culture collection at the University of Barcelona, Spain

Table 1 Strains used in the study in the order they were used in the microplate

Column No	Species	NCIMB no	Phylogenetic group
1	*Brevibacillus parabrevis*	7577	Gram +
2	*Brevundimonas diminuta*	9393	α-proteobacteria
3	*Citrobacter freundii*	12203	γ-proteobacteria
4	*Comamonas testosteroni*	8955	β-proteobacteria
5	*Curtobacterium sp*	10352	Gram +
6	*Delftia acidovorans*	9681	β-proteobacteria
7	*Kurthia gibsonii*	9758	Gram +
8	*Leucobacter komagatae*	13513	Gram +
9	*Pseudomonas aurantiaca*	10068	γ-proteobacteria
10	*Serratia rubidaea*	4	γ-proteobacteria
11	*Enterococcus saccharolyticus*	-	Gram +

12	Negative control		

Chemicals tested

Phenol, chlorinated phenols, and metal salts were of analytical grade, and purchased from various companies selling laboratory chemicals. The disinfectants were either of analytical grade and purchased from Sigma, or were ready to use solutions purchased from a pharmacy or directly from the retail sellers.

The following disinfectant/mixtures were tested:

Hydrogen peroxide – an oxidizing agent

Phenol – a chemical compound seldom used as disinfectant today due to its carcinogenic and other toxic properties

Chlorhexidine 1,6-bis (N5-p-chlorophenyl-N'-diguanido)hexane. Common in commercial disinfectants for pharmaceutical use

Triclosan (2-hydroxy-2',4,4'-trichlorodiphenyl ether) - a commonly used bactericide that is added to many consumer hygiene products, e.g soaps, hand lotions, toothpastes, and deodorants, as well as to fabrics and plastics (14).

Perasafe (http://www.viroderm.se/perasafe/perasafe.htm)

Perasafe is a mixture consisting mainly of sodium perborate (40-60%) and an alkylbenzenesulfonate (1-2%).

Virkon (http://www.viroderm.se/virkon/virkon.htm)

Virkon is a mixture consisting mainly of potassium persulphate (50%), sulfaminsyra (5%), hexametaphosphate (18%) and sodium dodecylbenzenesulphonate (15%)

Byotrol *2000* (http://www.byotrol.co.za/)

Byotrol is a mixture of different chemicals, including iso-thiazalones, ortho-phenyl phenol and quaternary ammonium salts

Test performance

In contrary to the previous study, which was performed on lyophilised micro-organisms (4), the present study was performed on fresh bacteria. The bacteria were first grown on nutrient agar at +28^{o}C for two days. A nutrient broth (LB-broth, Difco Labs.) containing 0.01% of the growth indicator 2,3,5-triphenyltetrazolium chloride (TZR, tetrazolium red, Sigma T-8877, St Louis, USA) was prepared. To each well in rows A – G of a round-bottomed 96-well microplate, 100 µl of this nutrient broth was added. The chemical to be tested was dissolved in the same broth, and 150 µl of the

solution was dispensed into each well of row H in the microplate. This solution was then serial diluted 1/3 in each of row G to B by transferring 50 µl between each row, after careful mixing.

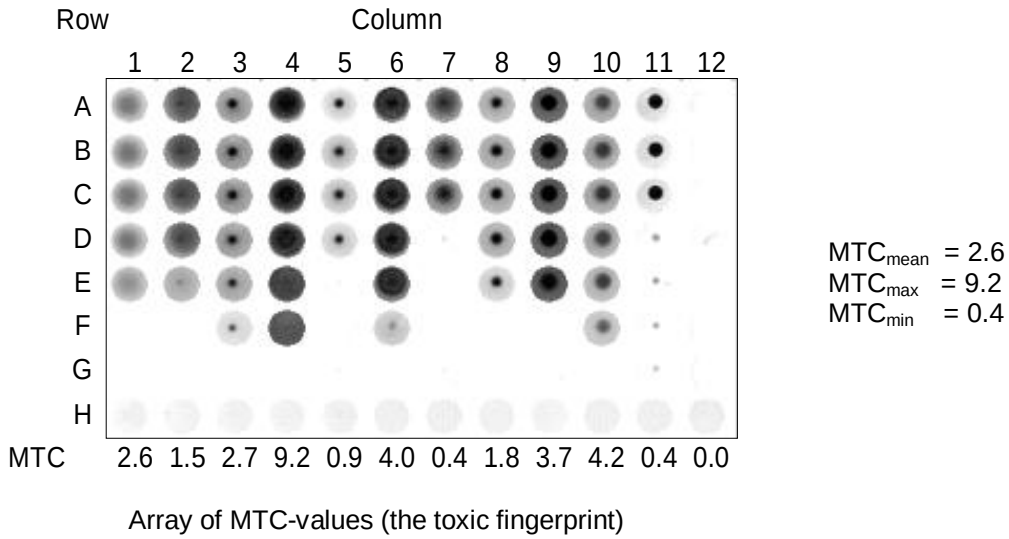
A loopful of each of the eleven bacterial strains was suspended in 5 ml of the nutrient broth. After carefully mixing, 10 µl of the bacterial suspensions were dispensed into all wells of column 1-11. The microplates were then incubated in 28°C for 24h in a wet chamber.

The microplates were scanned with a flatbed scanner that could read transparent originals (HP7400C), connected to a PC. The resulting scanned plate images were analysed with a specially developed software (available through PhPlate Microplate Techniques AB, Stockholm, Sweden, www.phplate.se) that estimated the size and the density of the pellets formed in the bottom of each well due to the bacterial growth (3).

Analysis of data

The Microbial Toxic Concentration (MTC) of the chemicals were calculated as the concentration of chemical yielding approximately 50% reduction of the bacterial growth, according to methods described before (4). The MTC values from the eleven bacterial strains obtained from each chemical thus formed an array consisting of eleven numerical values (Fig. 1). The mean MTC values were calculated as the average of the MTC values towards the eleven microbial strains (MTC_{mean}), and the maximum and minimum MTC (MTC_{max} and MTC_{min}) values were calculated as the concentrations that inhibited growth of the most resistant and the most sensitive strain, respectively. Arrays obtained with different chemicals (the toxic or microbial "fingerprints") were compared pair wise, and their similarities were calculated as correlation coefficients, yielding a similarity matrix, which was clustered according to the UPGMA method (15) to yield the dendrograms. All calculations were performed with the PhPWIN software (www.phplate.se).

Fig. 1. A MARA plate showing the toxic fingerprint obtained from eleven bacterial strains towards the disinfectant chlorhexidine. Bacterial strains 1-11 (see table 1) were dispensed into columns 1-11, column 12 was a control containing only the nutrient broth, indicator and the chlorhexidine gradient. Row H contains a concentration of 100 mg/L chlorhexidine, which was subject to serial dilutions of 1/3 in rows G-B. Row A was a control, containing bacteria without any addition of chlorhexidine. The MTC values are depicted below, for each bacterial strain, as well as mean, max, and min MTC values for all strains.



Results and discussion

Chlorinated phenols

The chlorinated phenols comprise a group of 19 chemical compounds consisting of phenol with one to five chlorine atoms. Pentachlorophenol and some tetrachlorophenols have been used worldwide, primarily as wood preservatives or fungicides. According to literature data, the acute toxicity of these chemicals is relatively low (2), and the acute oral LD50 in various species of animals ranges from 10-4000 mg/kg (which roughly corresponds to 0.05 – 20 mmol/kg) (16). In the present study, the effect of eleven different chlorophenols (also including unsubstituted phenol) on the eleven bacterial strains above was investigated. The resulting toxicity concentrations are shown in Table 2 together with literature data on toxicity concentrations from assays on goldfish (*Carassius auratus*).

Table 2. Chlorophenols tested with MARA, and toxic concentration for the eleven microbial strains shown as MTC_{mean}, MTC_{max}, and MTC_{min}. The last column gives the LC50-values for goldfish (*Carassius auratus*), as published by Kishino et al (8). Data are sorted in order of MTC_{mean} values

		MTC mmol/L			LC50 in
Chlorophenol	**Chlorine position**	**mean**	**max**	**min**	**goldfish***
Phenol	None	4.225	14.298	0.457	1.060
2,6-di	ortho-ortho	2.112	12.147	0.293	0.215
4-mono	para	1.705	12.093	0.188	0.234
2,4,6-tri	ortho-para-ortho	0.644	3.332	0.046	0.020
2-mono	ortho	0.381	1.791	0.032	0.721
3-mono	meta	0.333	1.217	0.061	0.389
2,5-di	ortho-meta	0.186	0.745	0.005	0.031
2,3-di	ortho-meta	0.071	0.169	0.016	0.068
3,4-di	meta-para	0.058	0.137	0.010	NA
2,3,4,5,6-penta	all	0.035	0.126	0.002	0.001
3,5-di	meta-meta	0.027	0.050	0.007	0.015

* mortality after 5h exposure in media containing the respective chemical

The MTC_{mean} values ranged from 0.03 to 4.2 mmol/L. When comparing MTC_{mean} values to the data from the test on goldfish, there was a quite good agreement (correlation between the assays = 0.71), and the toxic concentrations are only slightly higher in the MARA assay than in the goldfish assay. The MTC_{min} –values were even more sensitive than the goldfish assay for some of the chlorophenols.

According to studies on other organisms, the toxicity for chlorophenols generally increases with the degree of chlorination of the phenol ring (2). However, chlorophenols with chlorine in the 3 and 5 positions (meta chlorophenols) are often more toxic than expected solely on the basis of their number of chlorines (2). A study on the inhibiting effect on activated sludge of all 19 chlorophenols revealed that the most toxic chemical was not pentachlorophenol, with five chlorines, but the chlorophenols with 2-4 chlorines with two in the meta position were usually more toxic (ref till Beltrame et al, 1987). The results from MARA well follow these rules: The five most toxic chlorophenols were all those with at least two chlorines, and at least one in a meta position. The highest mean toxicity is expressed by 3,5-

dichlorophenol, which has both its chlorines in meta positions, and pentachlorophenol, which has five chlorines.
For *Daphnia* the LC50 of chlorophenols has been reported to be 0,3-6,0 mg/l (0.015 – 0.3 mmol/l) (16), and thus MTC_{mean} values in MARA were less sensitive than the Daphnia test. However, the MTC_{min} values were comparable to the Daphnia test.

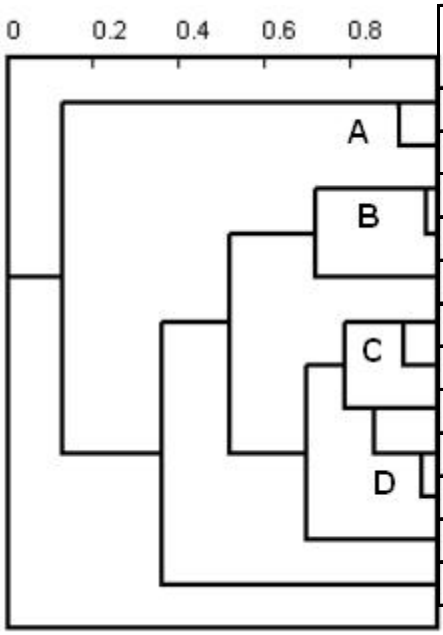
Another interesting parameter is the variation among the MTC values for a given chemical. For the chlorophenols this variation was between 65% (for 2,3-dichlorophenol) and 205% (for pentachlorophenol) (expressed as coefficient of variation, CV). It is possible that a low variation might indicate a more general toxicity, and that similar mechanisms of toxicity are expressed in different bacteria, as well as in other organisms, whereas a high variation indicates a more selective toxicity. Whether this is really the case has yet to be proven.

Clustering of toxic fingerprints of chlorophenols

The MTC-arrays for the chlorophenols were clustered to yield a dendrogram (Fig. 2). In some cases the chemicals yielded very similar toxic fingerprints, although they showed different levels of toxicity. This was the case for e.g. phenol and 2-monochlorphenol (cluster A), and for 4-mono and 3-monochlorophenol (cluster C). Such a result could indicate that the chemicals have similar modes of action, although their levels of toxicity are different. On the other hand, chemicals with similar levels of toxicity showed quite different toxic fingerprints (e.g. phenol and 2,6-dichlorphenol, or 2,3-dichlorphenol and pentachlorophenol), which means that although the average toxicity towards the eleven bacterial strains used was the same, the individual strains reacted differently. This could indicate that the chemicals differ in their modes of action on the bacteria.
The data presented here indicate a useful approach for dereplication of new compounds, e.g. for High Throughput Screenings (HTS) of chemicals that are candidates for drugs or other biological use. Of the chlorophenols showing similar toxic fingerprints, i.e. probably exert similar modes of action, only one chemical needs to be further investigated (that would probably be the one in each cluster showing the lowest toxicity, i.e. in cluster A this would be phenol, in cluster B 2,6-dichlorophenol, and in cluster C 4-monochlorophenol). Single chemicals that do not cluster with any other would also be further investigated. Thus this could be a useful

approach for companies that are developing new drugs or other chemicals, as well as for companies and authorities that need to assess the toxicity of old and new chemicals and consumer products.

Figure 2. Dendrogram derived from clustered toxic fingerprints of phenol and ten chlorophenols (including duplicate assays of 3,4-dichlorophenol). The scale on the x-axis denotes similarities, measured as correlation coefficients, between different fingerprints. Letters A-D in the dendrogram depict arbitrarily defined clusters of chemicals with similar toxic fingerprints



Chemical	Chlorine position	MTC_{mean} mmol/l
Phenol	None	4.20
2-mono	orto	0.38
2,6-di	orto-orto	2.11
2,4,6-tri	orto-para-orto	0.64
2,5-di	orto-meta	0.19
4-mono	para	1.71
3-mono	meta	0.33
2,3-di	orto-meta	0.07
3,4-di	meta-para	0.06
3,4-di	meta-para	nd*
3,5-di	meta-meta	0.03
penta	all	0.04

* 3,4-dichlorophenol was not properly dissolved on one assay occasion

Thus, the toxicity of the chlorophenols, given as MTC values, fit well with expectations according to their chemical structure, as well as with data from higher organisms, and the MTC arrays yield several clusters of chemicals that might represent differences in the mode of action of the chlorophenols.

Disinfectants

The disinfectants comprise a wide variety of chemical compounds and mixtures of compounds that are used for the same purpose, namely to kill bacteria and other micro-organisms. They are thus toxic to bacteria, but above certain concentrations often also to cells of higher organisms, including human and animal cells. In contrary to antibiotics, which preferably should kill only certain bacteria, the disinfectants

should affect a broad spectrum of micro-organisms. Disinfectants that can be applied on body surfaces are usually referred to as antiseptics.

Traditional disinfectants were chemicals that displayed a high general toxicity to microorganisms as well as to humans and animals. Examples are acids and alkalis, heavy metals, oxidizing agents (e.g. hydrogen peroxide and hypochlorous acid), alcohols and phenols. More modern disinfectants are often based on mixtures of different chemicals, which yield a broad spectrum of bactericidal action, without the need to use high concentrations of the individual components.

We have compared MTC arrays of some pure chemicals used as disinfectants with those obtained from commercially available disinfectants that contain mixtures of different active compounds (Table 3). In order to evaluate the reproducibility, some of the assays were also repeated at several occasions.

Table 3. The inhibitory concentrations of the tested disinfectants in the MARA assay. Average values from multiple assays were used when available.

Disinfectant	No. of assays	MTC mg/L			Recommended conc. mg/L	Reproducibility cv %
		mean	max	min		
Hydrogen peroxide	2	299	1070	19		2
Phenol	1	397	1344	43		
Chlorhexidine	5	2.01	6.03	0.15	200 - 5000	42
Triclosan	2	11	259	0.02	3000	46
Perasafe	4	720	2994	36	16000	5
Virkon	1	280	923	10	10000	
Byotrol	3	66*	233*	8*	NA	28
Triclosan + Chlorhexidine	1	1.02	3.169	0.0343		

* No information regarding the concentration of active ingredients could be obtained from the manufacturer. We have therefore assumed an arbitrarily concentration of 10% in the solution used

Perasafe showed the lowest toxicity, but according to the manufacturer, the concentration of the active ingredients in Perasafe is approximately 50%, and if this is taken into consideration, it would have a toxicity in the same range as hydrogen peroxide. Chlorhexidine seemed to be the most effective disinfectant, showing a MTC_{max} of only 6 mg/L. Chlorhexidine has also been shown to be more toxic also to

mammalian cells than many other disinfectants (6), and the high toxicity of this compound shown in MARA may thus be due to a high general toxicity.

The max and min MTC values may yield useful information. For disinfectants, the MTC_{max} values are an indication on the concentration necessary to affect more resistant bacteria, and the MTC_{min} values may be relevant for studies regarding environmental effects, since it gives an indication on the concentrations that would inhibit the growth of sensitive bacteria in the environment.

For triclosan, the MTC_{max} is more than 10 000 times higher than the MTC_{min} value. Pseudomonas and Serratia are often resistant to triclosan (13, Gibson 1978), a property that has been used for a selective medium for isolation of *Serratia spp* (Gibson 1978), and the *P.aurantiaca* and *S. rubidaea* strain used in the present study were also highly resistant (MTC_{mean} = 123 mg/L and 259 mg/L, respectively). Since both Pseudomonas and Serratia are bacteria that are not seldom encountered in infections in humans, it could be questioned whether triclosan really is suitable as a disinfectant. Another reason for concern is that triclosan may select for resistance towards clinically relevant antibiotics (1, 11).

The reproducibility of the MTC_{mean} values for disinfectants assayed at different occasions, measured as coefficient of variation, was below 50% in all cases. This corresponds to less than one dilution step difference between the highest ant the lowest MTC_{mean} value.
In order to visualize the relations between the toxic fingerprints of different disinfectants the MTC arrays were clustered according to the UPGMA method, resulting in a dendrogram (Fig. 3).

Perasafe and Virkon yielded similar toxic fingerprints, as well as MTC values in the same range, which could indicate that they have similar mechanisms of action. Since they both contain an oxidizing agent (Perasafe contains 40-50% of a perborate, Virkon contains 50% of a persulphate), this is not unexpected. Furthermore, they both contain the same kind of surfactant (an alkylbenzenesulfonate) as a secondary active ingredient. Hydrogen peroxide, another oxidizing agent, was also rather similar to these disinfectants.

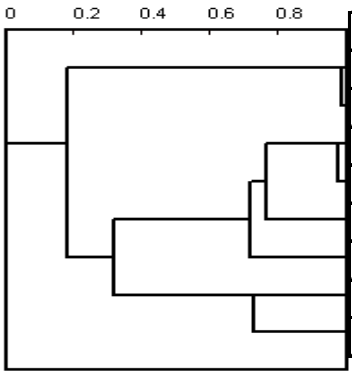
Triclosan and chlorhexidine both display very high MTC_{mean} towards the tested micro-organisms, but with totally different toxic fingerprints, which indicates

different mechanisms of action. Thus a combination of both chemicals would probably yield a high disinfecting effect at low concentrations. In fact, a mixture of triclosan and chlorhexidine 1:2 yielded an MTC_{mean} of 1.02mg/L and an MTC_{max} of only 3.2 mg/L (data not shown).

The toxic fingerprint of Byotrol shows a slight similarity to that of phenol. As inadequate data has been found regarding the composition of Byotrol, we can only speculate that it contains chemicals with a similar effect as phenol.

Two variants of chlorhexidine were included – one was a ready-made solution of chlorhexidine-acetate from the pharmacy, whereas the other was chlorhexidine-HCl purchased from a chemical company. Both these compounds showed almost identical mean MTC arrays, as well as identical toxic fingerprints. Also the MTC arrays of the other disinfectants that were subject to multiple assays showed high similarities when compared (always >0.92). Thus, the reproducibility of the clustered toxic fingerprints was good.

Figure 3. Dendrogram derived from clustering of the toxic fingerprints from seven disinfectants. Data from individual assays were used



Disinfectant	MTCmean mg/L
Chlorhexidine *	1.2
Chlorhexidine **	1.7
Perasafe	665
Virkon	280
Hydrogen peroxide	322
Triclosan	18
Byotrol	68***
Phenol	397

* Chlorhexidine acetate from the pharmacy
** Chlorhexidine-HCl from Sigma
*** Arbitrarily units

Many consumer hygiene products contain triclosan. We have tested two deodorants of world-wide well-known brands with MARA, and compared them to the results of pure triclosan. Fig. 4 shows three MARA plates that were exposed to concentration gradients of the three compounds.

No information was available on the contens of deodorant A, but since the MTC pattern shows low similarity to triclosan (correlation coefficient 0.01) it most probable does not contain this disinfectant. The MTC pattern of deodorant B shows a very high similarity to triclosan (correlation coefficient 0.98), and the toxicity is obviously very high to some of the bacteria. The mean MTC value of deodorant B is 0.01%, i.e. even if the deodorant that is to be applied to the body surface is diluted 10000 times it would still have a considerable bacteriocidal effect towards many bacteria. Compared to the pure chemical triclosan, the bacteriocidal effect of the deodorant corresponds to that of a concentration of triclosan of 5000 - 10000 mg/l, whereas most bacteria are killed by doses over 20 mg/ml (1).

Figure 4. Mara plates exposed to concentration gradients (3-fold dilutions) of different deodorants (A and B) and a solution of pure triclosan (C). Numbers in parenthesis denote the minimum and maximum concentration used for each plate. For A and B the concentration is calculated as percentage of the actual product.

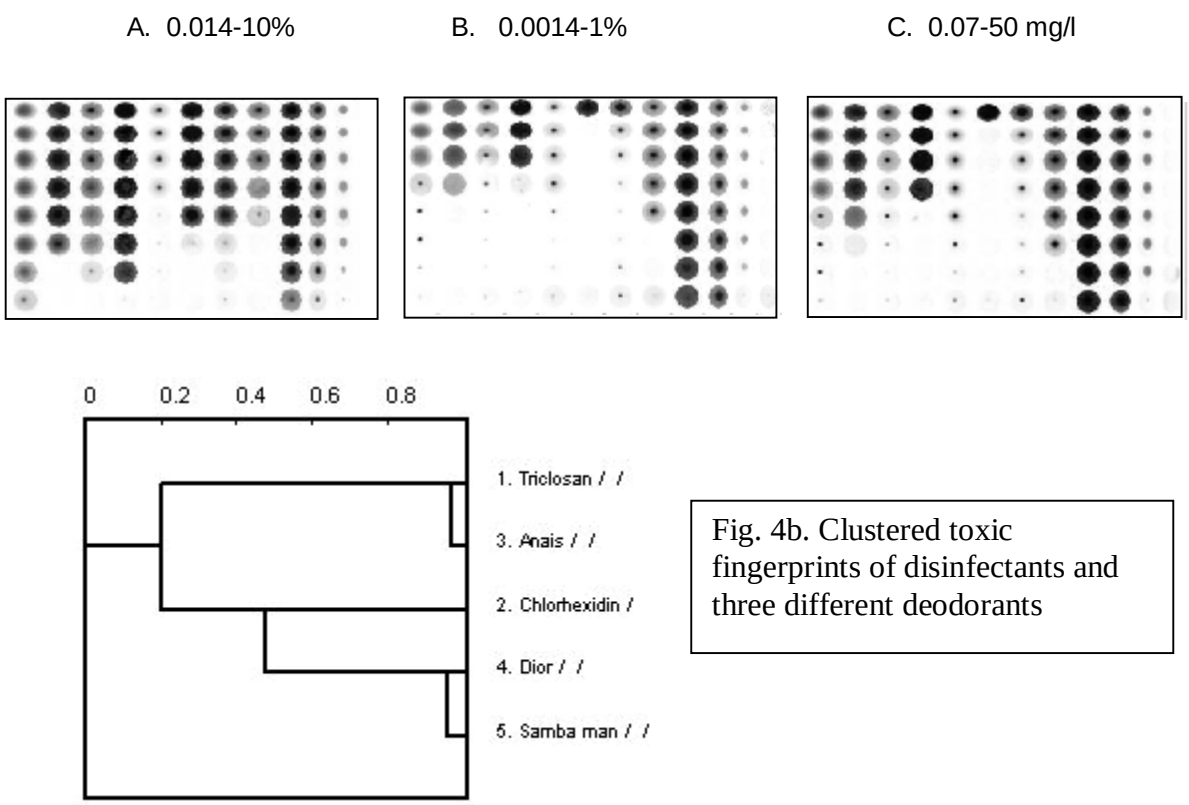


Fig. 4b. Clustered toxic fingerprints of disinfectants and three different deodorants

Thus, we have shown that MARA can be used to measure the effects of disinfectants in a standardised way. The mean, maximum, and minimum MTC values obtained from a standardized array of bacteria can give useful information on the concentration needed for a chemical to be an effective bug killer, and the toxic fingerprints may give useful clues to similarities or differences in the mechanisms of action, or to similarities of the components of different consumer products.

Metal salts

The metal salts are inorganic compounds that are involved in many biological processes, but that often may be toxic to living organisms, especially at higher concentrations. Fourteen different metal salts were evaluated with MARA, and the data, when available, were compared to toxicity data obtained from Microtox and the *Daphnia* test, as presented in the MEIC list (ref Bondesson samt till hemsidan) (Table 4).

Table 4. Metal salts tested with MARA, and toxic concentration for the eleven microbial strains shown as mg/L and as mmol/L. The last two columns show the IC50-values for Microtox and *Daphnia* assays. Data are sorted in order of MTC_{mean} mmol/L values

Chemical	MTC mg/L mean	MTC mmol/L mean	MTC mmol/L max	MTC mmol/L min	IC50 mmol/L Microtox	IC50 mmol/L *Daphnia*
KCl	21314	268	659	121	35	15
LiCl	10145	259	389	70		
Li2SO4	15730	148	333	51	234	1.8
MgCl2	20817	90	217	29		
NaF	2123	42	94	9.9	234	7.2
ZnOAc	877	4.5	13	0.54		
ZnCl3	509	4.4	10	0.37		
Pb(OAc)2	2427	2.7	16	0.17		
MnSO4	672	2.0	8.8	0.48		
FeSO4	657	1.1	3.3	0.16	8.5	0.09
FeCl3	633	1.0	4.0	0.09		
CuSO4	169	0.72	2.1	0.14	0.55	0.001
CoCl2	95	0.25	1.2	0.08		
AgNO3	7	0.03	0.09	0.01		

The MTC values of the tested metal salts showed high variations – from potassium chloride showing the lowest toxicity to silver nitrate, showing a toxicity that was almost 10000 times higher. The sensitivity of MARA was usually higher or comparable to that of Microtox, but lower than that of the *Daphnia* assay (Table 4). The span between MTC_{max} and MTC_{min} (calculated as MTC_{max}/MTC_{min}) for each tested metal salts was generally quite low (median value 15) compared to that for chloropenols (median value 41) and disinfectants (median value 56). This is an indication that the toxicity for individual metal salt is not very selective, and possibly that similar mechanisms of toxicity are effective in all organisms.
Clustering of the MTC arrays of the metal salts showed that they were quite homogeneous (data not shown). Only sodium fluoride showed a toxic fingerprint that totally differed from the other metal salts. This is probably due to the fact that the main toxic effect is expressed by the fluoride ion, rather than the sodium ion. The rather high toxicity of sodium fluoride (Table 4) compared to that of salts of other alcali metals further supports this assumption.

Conclusions

Different metal salts show high variations in their general toxicity in the MARA assay, but individual metal salts show rather low differenses in their toxicity towards different bacteria which indicate that the mode of action is similar to all micro-organisms

General discussion

The concept “fingerprinting” means to generate patterns of unknown organisms or items (e.g. humans, micro-organisms, or, as in the case of the present study, chemicals). As for a fingerprint of a human, the pattern itself contains no information about the organism (or chemical) to which it belongs, but is only useful when it is compared to patterns generated in the same way from other organisms. The concept of biochemical fingerprinting is often used for identification of clinically or hygienically relevant bacteria. It has traditionally been performed by exposing the strains to a set of chemical compounds (usually as different carbon sources that are added to a growth medium), and by measuring the responses of the bacteria to these compounds.

This results in a “biochemical fingerprint” for each strain that can be compared to and identified to biochemical fingerprints of other strains.

Once the unknown bacteria is identified, we do not only know the name of the bacterium, but also many of its properties, e.g. what kind of diseases it may cause, what treatment to use etc. One main innovative feature of MARA described in present study is the transfer of the principle of bacterial fingerprinting to produce “reversed” biochemical fingerprints, i.e. toxic fingerprints that will be created by exposing known micro-organisms to “unknown” chemicals. The hypothesis is that once the toxic fingerprint of an unknown chemical has been identified as similar to a known chemical, this might also produce information of many of the properties of the chemical, e.g. what kind of biological effects it may have. That this hypothesis is feasible has been shown in the examples presented here. It must be stressed that MARA is not intended to be an alternative to chemical analysis of e.g. consumer hygiene products, although it might yield some information on the contents of such products, as was visualised in Fig. 4, but more to yield an overview of the toxic effects of the products or chemicals. The information value of such data will increase as the experiences of MARA assays of products and chemicals increase. It is a vision for the future to extend MARA with more micro-organisms, and to combine MARA results with data obtained from other *in vitro* assays, and thus to generate toxic fingerprints that can give a reliable representation of toxic and other biological effects of old and new chemicals, thus diminishing the need for extensive toxicity testing using animals

Conclusions

The data presented here have shown that toxic fingerprinting with MARA yield results that seem to be in concordance with the chemical nature and the biological effects of the tested chemicals. Thus, MARA can be a candidate test when testing new chemicals to get an idea about their biological properties and modes of action.

Acknowledgements

This study has been performed with financial support from European Union’s Fifth Framework Program, project number MAS3-CT98-0181, Vinnova, project number

14239-3, The Swedish National Board for Laboratory Animals, and The Swedish Animal Welfare Agency, project number 34-329. A special thanks to our collegue, dr. Douglas McKenzie, for all his efforts as co-ordinator of the European project that made it possible to initiate this work

References

1. **Aiello, A. E., B. Marshall, S. B. Levy, P. Della-Latta, and E. Larson.** 2004. Relationship between triclosan and susceptibilities of bacteria isolated from hands in the community. Antimicrob Agents Chemother **48:**2973-9.
2. **Exon, J. H.** 1984. A review of chlorinated phenols. Vet Hum Toxicol **26:**508-20.
3. **Gabrielson, J., M. Hart, A. Jarelöv, I. Kühn, D. McKenzie, and R. Möllby.** 2002. Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. Journal of Microbiological Methods **50:**63-73.
4. **Gabrielson, J., I. Kühn, P. Colque-Navarro, M. Hart, A. Iversen, D. McKenzie, and R. Mollby.** 2003. Microplate-based microbial assay for risk assessment and (eco)toxic fingerprinting of chemicals. Analytica Chimica Acta **485:**121-130.
5. **Gabrielson, J., I. Kühn, R. de Karzov, P. Colque-Navarro, and R. Mollby.** 2004. Bacterial diversity related to the toxic impact of chemicals. In manuscript.
6. **Kalteis, T., C. Luring, J. Schaumburger, L. Perlick, H. Bathis, and J. Grifka.** 2003. [Tissue toxicity of antiseptics]. Z Orthop Ihre Grenzgeb **141:**233-8.
7. **Kim, B. C., K. S. Park, S. D. Kim, and M. B. Gu.** 2003. Evaluation of a high throughput toxicity biosensor and comparison with a Daphnia magna bioassay. Biosens Bioelectron **18:**821-6.
8. **Kishino, T., and K. Kobayshi.** 1996. Studies on the mechanism of toxicity of chlorophenols found in fish through quantitative structure-activity relationships. Water Research **30:**393-399.
9. **la Farre, M., M. J. Garcia, L. Tirapu, A. Ginebreda, and D. Barcelo.** 2001. Wastewater toxicity screening of non-ionic surfactants by Toxalert (R) and Microtox (R) bioluminescence inhibition assays. Analytica Chimica Acta **427:**181-189.
10. **Lajoie, C. A., S. C. Lin, H. Nguyen, and C. J. Kelly.** 2002. A toxicity testing protocol using a bioluminescent reporter bacterium from activated sludge. J Microbiol Methods **50:**273-82.
11. **Levy, S. B.** 2001. Antibacterial household products: cause for concern. Emerg Infect Dis **7:**512-5.
12. **Manzo, S.** 2004. Sea urchin embryotoxicity test: proposal for a simplified bioassay. Ecotoxicol Environ Saf **57:**123-8.
13. **Russell, A. D.** 2004. Whither triclosan? J Antimicrob Chemother **53:**693-5.
14. **Schweizer, H. P.** 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol Lett **202:**1-7.

15. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical Taxonomy. W H Freeman and Company, San Francisco.
16. **WHO working group.** 1989. Chlorophenols other than pentachlorophenol. Environmental Health Criteria **93**.
17. **Woese, C. R.** 1992. Procaryote systematics: The evolution of a science, The procaryotes, vol. 1.