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Analytica Chimica Acta 485 (2003) 121–130
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Microplate-based microbial assay for risk assessment and (eco)toxic fingerprinting of chemicals

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Received 14 October 2002; received in revised form 21 March 2003; accepted 26 March 2003

Abstract

We have developed a multi-species microbial assay, MARA, for assessing the (eco)toxic risks of chemical compounds and for the determination of their toxic fingerprints. The main advantages with MARA are (1) the simultaneous testing on several microbial strains; (2) the concept of toxic fingerprinting; (3) the simple and inexpensive handling and reading of the test. The toxic activity is measured in parallel on 11 different micro-organisms lyophilised in a microplate. A concentration gradient of the chemical to be tested is added and growth is indicated through the reduction of tetrazolium red (TTC). The microplates are read by a common flatbed scanner or a microplate spectrophotometer. The array of the 11 different inhibition values constitute a toxic fingerprint, characteristic for each type of chemical compound, and it is shown that the assay can distinguish between 12 standard chemicals. Both the reproducibility (CV $\approx$ 20%) and the sensitivity are similar to other toxicity tests based on micro-organisms.

Keywords: Micro-organism; Ecotoxicity testing; Growth inhibition; Bioassay; Multi-species; Toxic fingerprint; Toxkit

1. Introduction

The range of novel chemicals being produced for agricultural, industrial, domestic and pharmaceutical use is rapidly increasing. There is a considerable backlog in thoroughly testing all of these chemicals for their toxicity and (eco)toxicity. This is compounded by the need of better understanding, particularly of (eco)toxicity, and of better tools to deal with the volume of testing required.

Drug companies, faced with a rapidly increasing number of targets that drugs have to be screened against, have moved to methods of high throughput screening (HTS) [1,2]. Applying similar technologies to (eco)toxicity testing would improve the situation. As with drug screening, (eco)toxicity screening should be fast, inexpensive, standardised and globally applicable, easy to use and ethically acceptable. No currently available (eco)toxicity test fully matches all of these criteria.

The aim of an (eco)toxicity test is to predict the effects of, e.g. a chemical or effluent when being discharged in nature. Thus, the test should yield some sort of ecologically relevant data. This is seldom obtained

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doi:10.1016/S0003-2670(03)00404-5

with single-species tests [3], since very little can be said about the effects on a whole system from the data obtained from just one organism. The optimal test would be a real ecosystem; on a laboratory level this may be obtained by a controlled, space-limited ecosystem, a so-called mesocosm. A problem with such a system is that the amount of data generated may be very large and difficult to interpret [4]. Several studies [5,6] have shown that a combined set of single-species tests can be used to generate "safe" No Observed Effect Concentration (NOEC)-values for an ecosystem. Furthermore, a combination of rapid-screening toxicity tests may be suitable for use as a first-line estimate of toxicity of pure chemicals even though it cannot completely replace standard acute toxicity tests, such as tests on water fleas (*Daphnia Magna*) or green algae (*Selenastrum capricornutum*) [7].

There are some bacteria-based test systems on the market, such as Microtox, ToxAlert [8] and Polytox [9]. These systems have been used quite extensively, especially Microtox, but they all suffer from the drawback that they only return one value from each assay; Microtox and ToxAlert because they rely on one bacterial strain only and Polytox because a single result comes from a mixture of bacteria. Furthermore, they are rather cumbersome to use. Botsford et al. [10] and Bitton et al. [11] have presented alternative bacteria-based toxicity assays, but also these tests rely on one strain only (*Rhizobium meliloti* and *Echerichia coli*, respectively). However, the use of micro-organisms in (eco)toxicity assays offers a number of advantages over animal-based tests. High numbers of individual organisms can be used, coupled with short generation times that make the required testing time short. Micro-organisms are often tolerant to a much wider range of environmental parameters than animals. There are also far fewer ethical and regulatory problems. On the other hand, there are toxic effects that are revealed when chemicals are tested on the relevant species only and this limitation must be kept in mind when making toxicity tests on micro-organisms.

The aim of this study was to design and evaluate a multi-species, easy to use (eco)toxicity-assay based on micro-organisms (MARA—a Microbial Assay for Risk Assessment). The concept of the assay is to expose an array of microbial strains in a microplate to a concentration gradient of the chemical compound that is to be tested. A "toxic fingerprint" is obtained, consisting of the inhibition values from the 11 strains. We also evaluated the possibility to compare toxic fingerprints from different compounds using numerical methods.

2. Materials and methods

2.1. Microbial strains

The 11 microbial strains used in this study were known bacteria from culture collections and environmental strains of marine origin (Table 1). The strains

Table 1
Microbial strains used in the first generation MARA plates

Column in microplate	Species	Designation	Origin
1	*Pseudomonas fluorescens*	JG 11	Skagerrak
2	*Pseudomonas fluorescens*	JG 59	Blackan[a]
3	*Microbacterium trichothecenolyticum*	JG 66	Blackan[a]
4	*Pseudomonas fluorescens*	JG 110	Oban[b]
5	*Stenotrophomonas maltophila*-like, β-proteobacterium		Roslagen[a]
6	*Aeromonas hydrophila*		Swedish drinking water
7	*E. coli*	MZ 480	Mozambique
8	*Micrococcus* sp.	Oban F	Oban[b]
9	*Saccharomyces cerevisiae*	Baking yeast	Jästbolaget, Sollentuna, Sweden
10	*Aeromonas caviae*	BDE12	Pond in Bangladesh
11	*Staphylococcus* sp.		Baby skin
12	Blank		

[a] Swedish east coast, brackish water.
[b] Scottish west coast.

were selected after screening of about 150 different microbial strains, both from strain collections and unidentified strains of marine origin that had first been dereplicated with the Phene Plate-system [12]. The strains were tested for susceptibility, reproducibility, stability during lyophilisation, reaction with growth indicator and variability in their response to different chemicals.

2.2. Microplates

The microplates used in the assays were 96-well round bottomed plates of polystyrene (Greiner Labortechnik, Frickenhausen, Germany, Catalog No. 650101) unless otherwise indicated. In one assay plates of polypropylene were used (Greiner, Catalog No. 650201). The plates were aseptically packed, although not sterilised, and our tests showed that no externally contaminating microbial growth occurs in the wells after lyophilisation.

2.3. Lyophilisation procedure

A loopful bacteria, cultivated on Nutrient Agar plates containing beef extract, peptone and NaCl (Difco Labs. Detroit, USA), were added to 50 ml Falcon-tubes containing 5 ml nutrient broth (Difco Labs.). The tubes were incubated on a shaking-table overnight (2 rps, 28 °C). Bacterial cells were harvested by centrifugation on the following day (50 rps, 15 min, 90% break) and suspended in 3 ml lyophilisation media (2% dextran, 7.5% glucose and 0.03 M phosphate buffer pH 7.5). Thereafter, the suspensions were distributed into 96-well round-bottomed microplates, 12.5 μl/well, according to the layout in Fig. 1. The plates were placed in −70 °C over-night and subsequently placed into the lyophilisator (Leybold, Lyovac GT2), which was operated at 5×10^{-5} bar for 6 h. After lyophilisation the plates were packed in sealed aluminium bags filled with N_2-gas and a bag with a desiccating agent (silica gel). The plates were stored at −20 °C. At this temperature, no change of the sensitivity of the test was seen after 6 months of storage.

2.4. Assay procedure

To each well of the pre-prepared 96-well microplates, 100 μl of nutrient broth (Difco Labs.) mixed with a concentration gradient of the chemical to be tested and supplemented with 0.01% of the growth indicator 2,3,5-triphenyltetrazolium chloride (TZR, tetrazolium red, Sigma T-8877, St. Louis, MO, USA) was added (Fig. 1). The wells in the lyophilised microplates contained about 10^5 bacteria each. After inoculation the microplates were incubated in 28 °C for 24 h in a wet chamber.

The microplates were read with a flatbed scanner (Agfa 600, Agfa-Gevaert, Mortsel, Belgium), connected to a personnel computer according to the

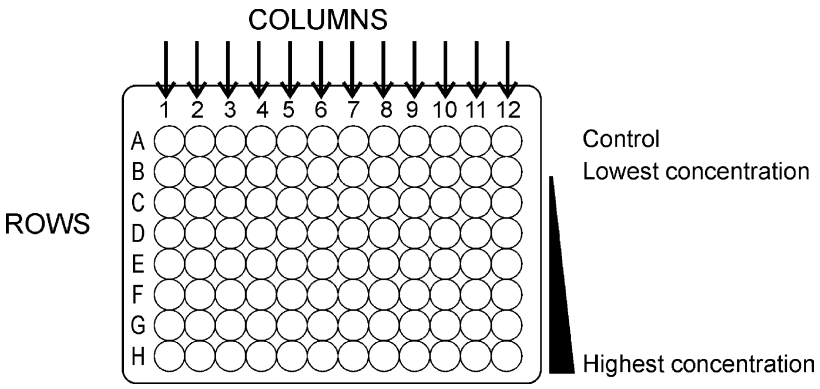

Fig. 1. A schematic picture of a MARA plate. Eleven different microbial strains are lyophilised in a 96-well microplate, one strain in each of column 1–11 as indicated by the arrows. Column 12 is a control. The chemical to be tested for its toxicity is added to the pre-prepared plate in a concentration gradient, the highest concentration in row H and the lowest in row B. Row A is the negative control where the bacteria may grow without interference of the tested chemical.

manufacturer's instructions. The amount (i.e. the diameter and the density) of the pellets formed in the bottom of the wells due to the bacterial growth, were measured with software developed in-house (available through PhPlate Microplate Techniques, Stockholm, Sweden, http://www.phplate.se) [13].

2.5. Computations

A new method for expressing the degree of inhibition was developed. The Microbial Toxic Concentration (MTC) values were calculated by the following formula

$$\mathrm{MTC} = c_{\min} d^{(P_{\mathrm{tot}}/P_0)-0.5}$$

where $c_{\min}$ is the lowest concentration in the gradient, P_0 the pellet size in the control well, d the dilution factor and P_{tot} the sum of the pellet sizes in all wells exposed to the concentration gradient of the chemical to be tested.

The MTC value is thus the concentration corresponding to the mean pellet size of each strain in the plate. As the pellet shapes differ between the strains, the size of the negative control is included in the formula. It is calculated automatically by the in-house software used to analyse the image of the scanned plates.

For comparison, three other computation methods for determination of the level of inhibition were also evaluated. The Lowest Observed Effect Level (LOEL) is the lowest tested concentration of the chemical at which a reduction of growth is observed as was measured by visual inspection of the plates.

The Minimal Inhibitory Concentration (MIC) is the lowest tested concentration of the chemical at which full inhibition of microbial growth is observed. Just as the LOEL value, it was measured by visual inspection of the plates in this study.

The IC_{50} value is the Concentration at which 50% Inhibition of the tested strain is obtained. It was calculated by making an interpolation between the pellets giving the values closest under and above 50% of maximum pellet size, assuming a linear relationship between the pellet size and the chemical concentration between these two measurement points.

An array of inhibition-values was obtained from each tested chemical compound with any of the above calculation methods. This array constituted the "toxic fingerprint" of the compound.

The toxic fingerprints obtained from chemicals tested in MARA plates were compared pair wise and their similarities were calculated as correlation coefficients. The resulting similarity matrix was clustered according to the Unweighted Pair Group Method using Arithmetic Averages (UPGMA)-method [14] to yield a dendrogram. In the dendrogram, the chemicals are connected with vertical lines according to the similarities between their toxic fingerprints, which can be read on the *x*-axis. Calculations of similarities and clustering were made with the PhP software from PhPlate Microplate Techniques.

3. Results

The MARA system in the present study consists of an array of 11 microbial strains in a 96-well microplate that is exposed to a concentration gradient of a chemical to be tested (Fig. 1). Fig. 2 shows an example of a MARA plate to which a concentration gradient of hydrogen peroxide (H_2O_2) was added. Replicates ($n = 10$) of the assay in Fig. 2 were performed and the inhibition value for each strain was calculated and summarised in Table 2.

A growth response pattern is obtained in the MARA plate due to the varying susceptibilities to the added chemicals exhibited by each individual strain. This pattern, the "toxic fingerprint", varies for different chemicals, as shown in Fig. 3. The toxic fingerprints obtained from Fig. 3 are shown as MTC values in bar charts (Fig. 4). The system is apparently discriminative enough to distinguish between the four tested chemicals according to their microbial responses, which is also shown in the dendrogram in Fig. 5.

In order to compare the sensitivity of the MARA test to that of other toxicity tests, 13 chemicals from which toxicity data from a number of in vitro assays were already available from the Multicentre Evaluation of In vitro Cytotoxicity (MEIC) list [15] were tested in MARA plates. The chemicals were selected according to their availability and easiness to handle rather than their (eco)toxic effects. The MTC values from the MARA test were compared to the results from three other assays in the MEIC list, namely Microtox (based on the luminescent bacterium *Vibrio fischerii*), *Daph-*

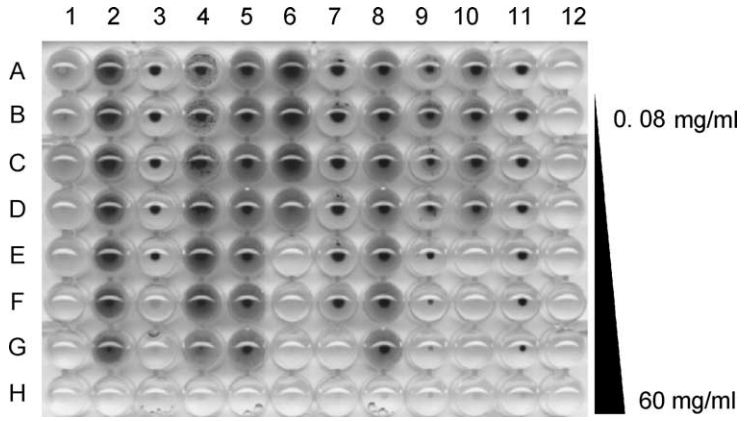

Fig. 2. MARA plate with a concentration gradient of H_2O_2 (0.08–60 mg/ml) from row B to row H. Layout as in Fig. 1 and strains as in Table 1. The black dots are due to the precipitated TTC, which indicates microbial growth. The figure shows that the different strains display different susceptibilities to H_2O_2.

Table 2
Comparison of four different methods (LOEL, MTC, IC_{50} and MIC) for measurement of the inhibitory effect of H_2O_2 on the micro-organisms in the plate in Fig. 2

	1[a]	2	3	4	5	6	7	8	9	10	11	Mean CV	CV range
LOEL	ND	5.80	2.95	7.13	28.0	0.69	10.2	36.0	1.47	1.62	7.58	0.60	0.34–0.87
MTC	ND	13.8	2.96	10.7	14.7	0.80	7.76	20.8	4.66	0.99	9.56	0.20	0.13–0.34
IC_{50}	ND	24.8	4.75	16.6	31.7	1.20	12.9	37.4	5.94	1.50	21.2	0.18	0.09–0.33
MIC	ND	56	7.94	60	60	2.2	20	60	56	2.2	60	0.10	0–0.53

The mean values of the inhibitory concentrations ([mg/ml]) from 10 plates are given for each strain. The mean CV value expresses the reproducibility.

[a] Data from column 1 are not included due to lack of growth of this strain.

nia and *E. coli*. (Table 3). The MARA test generated results that are well within the range of the sensitivity of the other tests. The exception is potassium chloride, where MARA showed a much lower sensitivity than the other assays. An explanation to the lower sensitivity could be that many of the strains in the MARA plate are of marine origin and are thus used to a higher level of Cl^- ions. The MTC values are presented in a dendrogram (Fig. 6) which shows that duplicate samples cluster together and that the MARA test is discriminative enough to separate all tested chemicals except for the two drugs quinidine sulphate and orphenadrine HCl, which yielded similar toxic fingerprints.

4. Discussion

The aim of this project was to develop a cheap, simple and easy to use (eco)toxicity test based on an array of micro-organisms, mainly bacteria. The concept of a multi-species assay based on micro-organisms is not all together unique. A similar principle was used by Eloff [16] and Mattila [17] for determination of antimicrobial activity of plant extracts and disinfectants, respectively. The tests were performed on four different bacterial species in microplates and growth was detected with tetrazolium salts. Other methods are based on genetically modified bacteria, e.g. a panel consisting of five strains of luminous *E. coli*, each genetically engineered to respond to different types of stress [18].

In comparison to these methods, four new features are introduced with the MARA method: (1) the concept of the toxic fingerprint based on the result from an array of microbial strains belonging to different species. (2) The use of pattern recognition, based on the comparison of the toxic fingerprint from a tested compound to a database consisting of fingerprints from

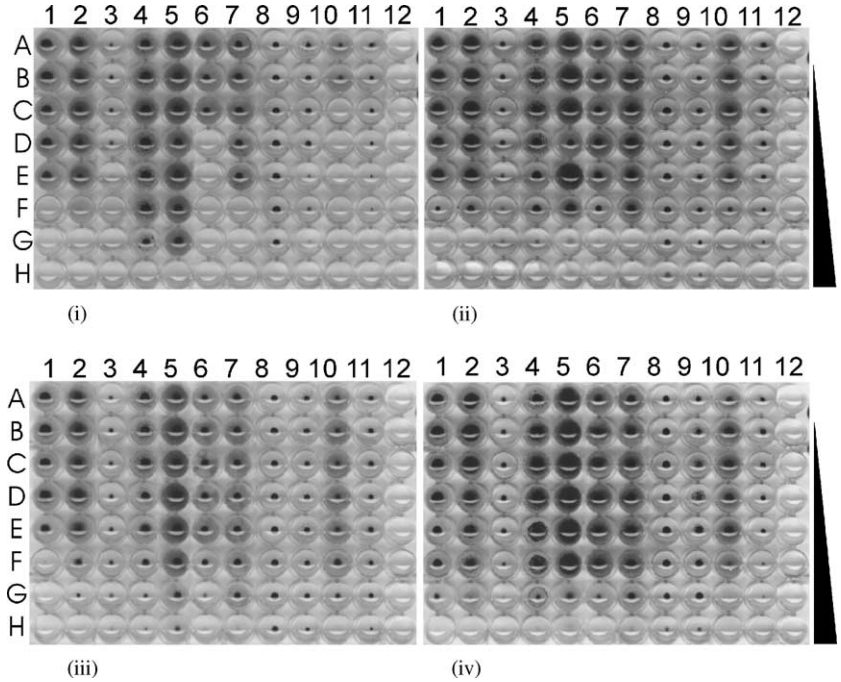

Fig. 3. Four MARA plates that have been incubated with four different chemicals using a three-fold dilution of the chemicals in each step from row B to row H. Layout as in Fig. 1 and strains as in Table 1. The black dots are due to the precipitated TTC, which indicates microbial growth. The different strains display different susceptibilities to each chemical and each strain display different susceptibilities to different chemicals. Thus, a unique growth pattern, the toxic fingerprint, for each chemical is obtained: (i) H_2O_2, 0.08–60 mg/ml; (ii) phenol, 0.014–10 mg/ml; (iii) acrylamide, 0.041–30 mg/ml; (iv) glyphosphate, 0.016–12 mg/ml.

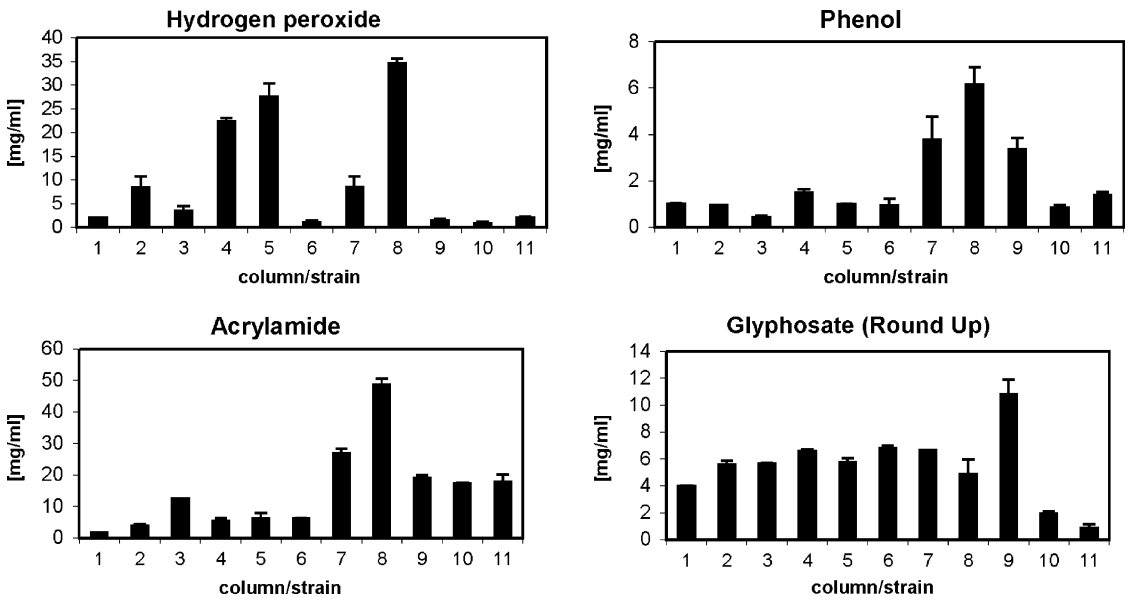

Fig. 4. Bar charts depicting the toxic fingerprints of the four chemicals tested in Fig. 3. Each bar represents the mean MTC value ± S.D. ($n = 2$) of one strain. The variations in fingerprints are easily distinguished by eye.

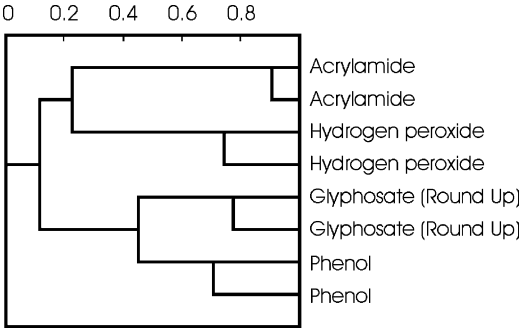

Fig. 5. Dendrogram showing clustered toxic fingerprints, based on MTC values, of the four chemicals tested in Fig. 3. Each assay in Fig. 3 was done in duplicate. The duplicate assays of the four chemicals cluster together, indicating a good reproducibility of the assay. The dendrogram also visualises the differences between the toxic fingerprints of the four chemicals.

a large number of previously tested compounds with known toxic properties. (3) The new method for calculation of growth inhibitory concentrations, Microbial Toxic Concentration (MTC). (4) The simple reading of the result. A common flat-bed scanner combined with an especially developed software are sufficient devices [13].

The combination of these features could make the MARA test a powerful alternative in acute (eco) toxicity testing as well as in other toxicity screenings. It has a number of advantages compared to existing assays (Microtox, Polytox, etc.), such as the standardised simultaneous testing on several organisms and the easiness to use. By using pre-fabricated microplates with lyophilised bacteria, a high degree of standardisation as well as a simple performance may be obtained with the MARA test. The amount of time required for the preparation of the test of a chemical in seven different concentrations is less than 30 min and thus a number of tests may be performed simultaneously. Even though a test based on micro-organisms can never fully replace tests on higher organisms as they only represent one trophic level, it can be very useful for screening purposes.

One advantage of using bacteria instead of higher organisms in multi-species toxicity testing is their wide genetic diversity. It is considerably larger than in the eucaryotes [19]. A wide genetic diversity of the micro-organisms in the MARA test is important, but even more important is to select strains that yield a high diversity in their responses to the chemicals to be tested. Three strains of the species *Pseudomonas fluorescence* (Table 1) were selected because they were among the most diverse in their response to different chemicals among the 150 tested strains; on the other hand *Pseudomonas fluorescence* consists

Table 3
Comparison between the MARA test and results from other toxicity tests

MEIC no.		Microtox	Daphnia	*E. coli*	MARA	
					Mean	Min.
7	Ethylene glycol	167	75	271	48	14
12	Phenol	0.13	0.007	2.3	1.4	0.24
14	Sodium fluoride	9.8	0.64	11	14	3.8
20	Lithium sulphate	26	0.03	68	19	4.5
29	Thioridazine HCl	0.01	0.005	0.06	0.048	0.011
37	Barium nitrate	29	0.21	ND[a]	2.4	0.35
39	Pentachlorophenol	0.0006	0.0006	0.01	0.009	0.0007
40	Verapamil HCl	0.44	0.06	0.83	1.4	0.23
42	Orphenadrine HCl	0.12	0.011	0.70	0.24	0.047
43	Quinidine sulphate	0.09	0.06	1.0	0.26	0.092
48	Caffeine	2.1	0.16	15	3.6	0.92
49	Atropine sulphate	3.1	0.35	56	6.9	0.92
50	Potassium chloride	2.6	0.55	1.4	52	19

The results for Microtox, *Daphnia* and *E. coli* were collected from the MEIC-list. The results are given as EC_{50} values (EC: effective concentration) for Microtox and *Daphnia*, as MTC values for the MARA test, and as Minimal Inhibitory Concentration values (MIC) for *E. coli*. All values are given in mg/ml.

[a] ND: no data available.

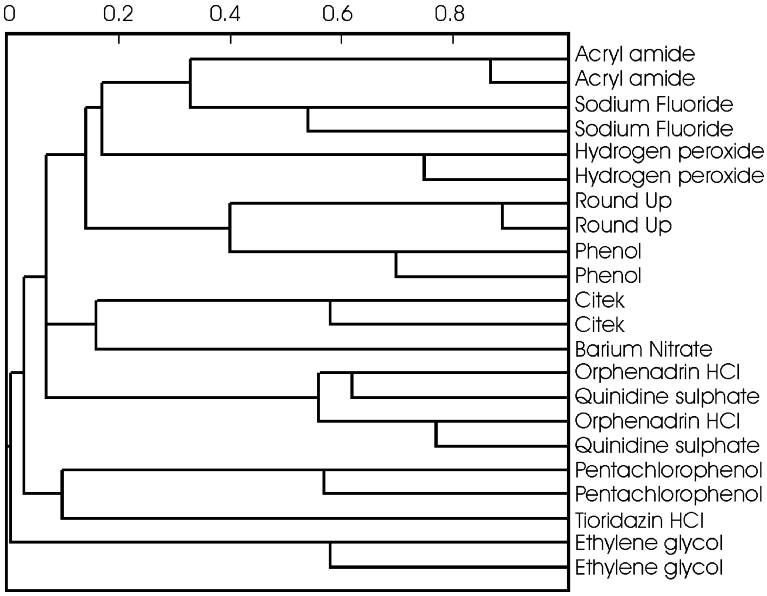

Fig. 6. Dendrogram showing clustered toxic fingerprints, measured as MTC values, of 13 chemicals. Duplicate assays of the same chemical cluster together (e.g. phenol) whilst single assays come out separately (e.g. barium nitrate). Only quinidine sulphate and orphenadrine HCl could not be separated in the assay. Both chemicals act as P450 inhibitors, which can explain their similar fingerprints.

of a number of not very closely related biovars [20]. In the future, more microbial strains need to be screened to optimise the MARA plate and possibly more than one set of strains may be used depending on the type of test that is to be done. The dendrogram in Fig. 6 shows that the discriminative power of the system with the present set of micro-organisms is high enough to distinguish between most of 13 standard chemicals. The only two chemicals that could not be distinguished in the present example, orphenadrine HCl and quinidine sulphate (Fig. 6), are both cytochrome P450 inhibitors in eucaryotic organisms [21,22]. This indicates that chemicals with similar toxic fingerprints on micro-organisms may exhibit similarities in their mode of action in higher organisms as well. Thus, further development and evaluation of the MARA system will aim to make it possible to do predictions not only about the toxicity levels of tested compounds, but also about the type of toxicity they exhibit in higher organisms.

As the MARA plate generates 11 independent values from 11 microbial strains, a test result can be presented either as the mean inhibition value or as the minimum inhibition value if a single numerical value is required (Table 3). The mean value is probably a more “realistic” value, a common criticism against existing bacterial assays is that the result from one strain only is not representative. On the other hand, the minimum value is a valuable result as it shows at which concentration level the most susceptible species may be affected. However, most of the information gained in a multi-species test is lost if a single value is used for evaluation instead of the whole toxic fingerprint. Additional information can be obtained from the standard deviation of the 11 inhibition values. It gives information about whether the toxicity is general (low standard deviation) or specific (high standard deviation).

Four different methods to calculate and express the inhibition of the microbes have been compared in this study; LOEL, MIC, IC_{50} and MTC. The LOEL and the MIC values were obtained by visual reading, and they are thus easy to obtain but are fairly arbitrary. In addition, the only possible results when dealing with LOEL or MIC values are the concentrations actually used in the wells of the microplate. This decreases the resolution. The IC_{50} value is the concentration at which a 50% inhibition of the microbial growth (compared to the control well without any added chemical) is

obtained. To get a correct value, the shape of the inhibition curve should be known though since an interpolation must be performed to obtain the IC_{50} value. This is seldom the case, in this study we have found that the curve varies both between different microbial strains and between different chemicals. The MTC value is calculated by comparing the areas under and above the curve and thus the shape of the curve is irrelevant. The reproducibilities expressed as CV values (Table 2) gave by hand that LOEL yielded the lowest reproducibility (mean CV = 60%). MTC and IC_{50} yielded similar reproducibilities (mean CV = 20%) and similar ranges of CV values. The MIC value yielded the highest reproducibility (mean CV = 10%), but gave the largest range of CV values. A CV of 20% is at the same level as reproducibilities reported for other microbial-based toxicity assays [10,23].

The MARA method is based on the measurements of bacterial growth inhibition due to exposure to different toxicants. Other bacteria-based systems measure other parameters, such as ATP production or enzyme activity [24]. Torslov showed that the choice of parameter to be measured is just as important as the selection of strains for the result [25]. We have chosen to measure growth inhibition in the MARA test, both for the sake of simplicity and for the sake of covering a broad range of toxic mechanisms as most toxic mechanisms give reduced growth rate in the end. Due to the addition of a growth indicator, tetrazolium red (TTC), the plates may be read both with a flat-bed scanner or a microplate spectrophotometer [13]. The addition of TTC also enhances the sensitivity of the test. A scanner is a suitable reading device for end-users that do not have access to a spectrophotometer as it is considerably cheaper and easy to connect to a computer. A digital camera may also be used to read the result.

Microplates have been used for toxicity testing on various organisms in a number of studies. Gellert and Stommel [26] made a comparison between the effect of both pure chemicals and industrial effluent on bacteria cultivated in microplates of polystyrene and quartz glass, respectively. It was concluded that plates of quartz glass are preferable to plates of polystyrene if substances of hydrophobic character are tested as the chemicals may interfere with the plastic and thereby decrease the susceptibility of the assay. For an assay that is to be used commercially, plates of quartz glass are less suitable as the cost of one plate is about 1000 times the cost of a polystyrene plate. Plates of polypropylene would constitute a better alternative. They have a higher chemical resistance and they are still available at a reasonable price (about double the price of a polystyrene plate). We have compared polystyrene plates to plates of polypropylene and our results show that the polypropylene plates work well in lyophilisation with the media developed within the frames of this project and that they have the same sensitivity but a higher reproducibility (lower CV) than the polystyrene plates (data not shown). A plausible explanation to the higher reproducibility is that the polypropylene plates are more opaque than the polystyrene plates. Thus, the scanned pictures are more blurred and minor disturbances in the pellet shapes do not affect the result to the same extent.

5. Conclusion

We have shown that our multi-species Microbial Assay for Risk Assessment (MARA) can be used for acute (eco)toxicity testing. The result from a MARA test can be either a single value, as in most other tests, or a toxic fingerprint of the tested sample, which seems to be specific for individual chemical compounds. The MARA method appears to have a good discriminating power and a reproducibility and a susceptibility that is comparable to other similar methods.

Acknowledgements

This study has been performed with financial support from European Union's Fifth Framework Program, project number MAS3-CT98-0181, and The Swedish National Board for Laboratory Animals. A special thanks to Terry Dando, NCIMB, Scotland and Görel Allestam, Swedish Institute for Infectious Disease Control, for helping us identify the unknown strains and to Åke Hagström and Ulla Li Zweifel, Kalmar University, Sweden, for providing us with strain AF025327 (*Stenotrophomonas maltophila*-like).

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