An assessment of the potential of the microbial assay for risk assessment (MARA) for ecotoxicological testing

Patricia Bi Fai · Alastair Grant

Abstract Rapid microscale toxicity tests make it possible to screen large numbers of compounds and greatly simplify toxicity identification evaluation and other effect directed chemical analyses of effluents or environmental samples. Tests using *Vibrio fischeri* (such as Microtox®) detect toxicants that cause non-specific narcosis, but are insensitive to other important classes of contaminants. The microbial assay for risk assessment (MARA) is a 24 h multi-species test that seeks to address this problem by using a battery of ten bacteria and a fungus. But there has been little independent evaluation of this test, and there is no published information on its sensitivity to pesticides. Here, we assess the performance of MARA using a range of toxicants including reference chemicals, fungicides and environmental samples. Mean MARA microbial toxic concentrations and IC_{20}s (20% Inhibitory concentrations) indicate the toxicant concentrations affecting the more sensitive micro-organisms, while the mean IC_{50} (50% Inhibitory concentration) was found to be the concentration that was toxic to most MARA species. For the two fungicides tested, the yeast (*Pichia anomalia*) was the most sensitive of the ten MARA species, and was more sensitive than the nine other yeasts tested. The test may be particularly valuable for work with fungicides. Mean MARA IC_{50}s were comparable to values for nine other yeast species and the lowest individual IC_{50}s for each toxicant were comparable to reported IC_{50}s for *Daphnia magna*, *Selenastrum capricornutum* and Microtox® bioassays. MARA organisms exhibited more variable sensitivities, with the most sensitive organism being different for different samples, enhancing the likelihood of toxicity detection and giving a toxicity "fingerprint" that may help identify toxicants. The test, therefore, has great potential and would be valuable for ecotoxicological testing of pollutants.

Keywords Bacteria bioassays · Fungicides · Yeast growth inhibition · Microscale toxicity tests · Tetrazolium red

P. B. Fai (✉)
Department of Animal Biology, Faculty of Sciences,
University of Dschang, Dschang, West Region, Cameroon
e-mail: asangapb@yahoo.com

A. Grant
Centre for Ecology, Evolution and Conservation (CEEC),
School of Environmental Sciences, University of East Anglia,
Norwich NR4 7TJ, UK

Introduction

A range of acute toxicity bioassays have been developed to establish the ecotoxicological impact of contaminants on aquatic organisms (Farre and Barcelo 2003; Wadhia and Thompson 2007). However, many of the standard bioassays are still laborious, time-consuming and require large sample volumes (Parvez et al. 2006). The EU REACH regulation (*R*egistration, *E*valuation, *A*uthorisation and Restriction of *Ch*emical substances) requires industry to assess risks from, and provide safety information on, the great majority of manufactured chemicals. Rapid, easy to use, microscale ecotoxicity techniques would greatly contribute to the detection of aquatic pollution and the large-scale screening of compounds for toxicity required by regulatory risk assessment and toxicity identification evaluation (Wells et al. 2001). Therefore, short-term toxicity assays based on the inhibition of enzymes or micro-organisms such as bacteria, fungi, algae and protozoa, are of growing importance to assess the toxicity of chemicals, due to their simplicity, rapidity, cost-effectiveness and

reproducibility (Cascorbi et al. 1993; Ribeiro et al. 2000; Papaefthimiou et al. 2004). They also contribute to minimizing higher animal use in toxicity studies (Cabral et al. 2003).

Bacteria and yeasts are suitable organisms for ecotoxicity testing due to their small size, rapid growth rate, short generation time and ease of culturing and handling (Gabrielson et al. 2003; Parvez et al. 2006; Wadhia and Thompson 2007). They also incorporate toxicants in their metabolism faster than higher organisms do and ethical issues associated with higher organisms are not a concern (Gabrielson et al. 2003; Wadhia et al. 2007). Several bacterial bioassays have been developed, with Microtox® being most widely used. This is based on the inhibition of bioluminescent bacterium, *Vibrio fischeri* (Brack 2003; Gabrielson et al. 2003; Parvez et al. 2006; Farre et al. 2007; Greenwood et al. 2007; Wadhia and Thompson 2007; Ocampo-Duque et al. 2008). Short-term tests with *V. fischeri* detect effects on bacterial energy metabolism and are very sensitive to a range of toxicants and environmental samples (Parvez et al. 2006; Ocampo-Duque et al. 2008). However, although it detects log K_{OW}-dependent general narcosis, many ecotoxicologically relevant specific effects (e.g. of herbicides, insecticides, and antibiotics), and long-term effects, are hardly detectable in this test system (Reteuna et al. 1989; Brack 2003). So no single test system can satisfy the need to assess the quality of the aquatic ecosystem resulting from the multiplicity of both pollutants and effects (Benfenati et al. 2003). This suggests that the application of a battery of biotests using bioindicator species at different trophic levels will enhance both the chances of toxicant identification and the significance for hazard assessment of aquatic ecosystem (Cascorbi et al. 1993; Fochtman et al. 2000; Ribeiro et al. 2000; Brack 2003; Farre and Barcelo 2003; Manusadzianas et al. 2003; Pessala et al. 2004; Wadhia and Thompson 2007). Fochman et al. (2000) separated unicellular organisms from multicellular ones with regard to their sensitivity to pesticides and this led them to select a test battery consisting of Algaltoxkit F™ (*Raphidocelis subcapitata*), Protoxkit F™ (*Tetrahymena thermophila*), *Daphnia magna* (Fluotox), and *Oncorhynchus mykiss* or Thamnotoxkit F™ (*Thamnocephalus platyurus*). However, carrying out a test battery such as this would involve buying several test kits and carrying out several different tests, which will be more expensive, laborious, time-consuming and require more laboratory space.

The MARA is a recently developed 24 h multi-species test consisting of 11 taxonomically diverse microbial strains lyophilised (freeze-dried) in a 96-well microplate and used to assess the ecotoxicity of chemicals and environmental samples (Gabrielson et al. 2003; Wadhia et al. 2007; Wadhia and Thompson 2007). Growth of the micro-organisms is assessed by measuring the reduction of the redox dye, tetrazolium red (TTC). The strains show different sensitivities to different chemicals and the resulting array of 11 inhibition values gives a toxic fingerprint of the chemicals tested, providing a battery of tests within a test (Gabrielson et al. 2003; Wadhia and Thompson 2007).

One reason for the limited inclusion of microbiotests in the regulatory framework may be the lack of relevant published data for many of these tests (Wadhia and Thompson 2007). The sensitivity of the MARA test for a number of chemicals and environmental samples compares favourably with the *D. magna* and Microtox bioassays (Gabrielson et al. 2003), but there are no published data on MARA toxicity values for pesticides and many other important environmental pollutants. This study assesses the sensitivity of the MARA test to some reference toxicants, fungicides and environmental samples, and compares its sensitivity with that of a growth inhibition assay using nine phylogenetically diverse yeast species to determine its usefulness in effect-directed analysis of environmental contaminants.

Materials and methods

Materials

All materials and equipment for the MARA test were supplied by the UK National Collection of Industrial, Food and Marine Bacteria (NCIMB, Bucksburn, Aberdeen) and consisted of MARA plates (containing lyophilised MARA micro-organisms), microbial growth medium (phytone peptone), dye (1% w/v TTC solution), HP ScanJet 7400c (Wood Street, London) and MARA Software. The tested samples included two fungicides (captan and maneb), two reference toxicants (3,5-dichlorophenol and potassium dichromate), and two environmental samples (an industrial effluent and a soil leachate). The reference toxicants and environmental samples were supplied in aqueous solutions by NCIMB. Maneb was acquired as PESTANAL® analytical standard from Sigma-Aldrich (New Road Gillingham, Dorset, UK), while captan was obtained from Dr Ehrenstorfer GmbH (Augsburg, Germany). Details of the nine yeast species (*Schizosaccharomyces pombe, Saccharomyces cerevisiae, Lipomyces tetrasporus, Botryoascus synnaedendrus, Kluyveromyces marxianus, Pseudozyma fusiformata, Trichosporon dulcitum, Sporidiobolus pararoseus,* and *Cryptococcus laurentii*) used in the present study are found in our previous published work (Fai and Grant 2009a). All yeast species were obtained from the National Collection of Yeast Cultures (NCYC—Norwich, UK) and cultured at 30°C in liquid Yeast (1%) Peptone (2%) Dextrose (2%) (YPD) medium prepared according to

Treco and Lundblad ([1996](#)) and the pH adjusted to 7.0 using 0.165 M 3-(N-morpholino) propanesulfonic acid. Yeast extract was bought from Duchefa (Haarlem, The Netherlands), peptone from Fisher Scientific (Bishop Meadow Road, Loughborough, Leicestershire UK) and dextrose from Sigma-Aldrich (New Road Gillingham, Dorset, UK). The Spectra Max® M2 microplate reader from Molecular Devices Corporation (Sunnyvale, CA, USA) was used to measure absorbance for the yeast growth inhibition bioassay.

Methods

MARA

The MARA test was performed according to the standard protocol described by Wadhia et al. ([2007](#)) and Wadhia ([2008](#)). In the present work, the microbial species used consisted of ten bacterial species: *Microbacterium sp.*, *Brevundimonas diminuta*, *Citrobacter freundii*, *Comamonas testosteroni*, *Enterococcus casseliflavus*, *Delftia acidovorans*, *Kurthia gibsonii*, *Staphylococcus warnerii*, *Pseudomonas aurantiaca*, *Serratia rubidaea*, and one yeast species, *Pichia anomalia* (Wadhia [2008](#)). The numbers 1–11 were used to identify the 11 MARA species listed above. Three plates were used for each toxicant/sample tested, giving three replicates/toxicant/sample. Each plate contained positive (micro-organisms present, but no toxicant), and negative (no micro-organisms) controls. At the end of the test, the plates were scanned with a flatbed scanner using transmitted light, and the plate images transferred to Microsoft Excel files for storage until analysis.

Yeast growth inhibition bioassay

The yeast growth inhibition bioassay was carried out according to Papaefthimiou et al. ([2004](#)) with minimal modifications as reported by Fai and Grant ([2009a](#), [b](#)).

Statistical analysis

Statistical analyses were carried out using the MARA software, Microsoft Excel® and SPSS® 12.0.1 for Windows® with graphs plotted in Sigma Plot 2000.

The MARA plate images were copied from Excel files and loaded one at a time into the MARA software for analyses. The MARA software assigned numerical values to each well in the plate, which represented the amount of microbial growth. The software also calculated the percentage growth and growth inhibition of micro-organisms in each well relative to the respective controls as well as

MTC values for each organism and an overall mean MTC for the 11 MARA organisms in the plate (Gabrielson et al. [2003](#); Wadhia et al. [2007](#)). The MTC is calculated by the MARA software according to the formula $MTC = c_{min} d^{(P_{tot}/P_0)-0.5}$, developed by Gabrielson et al. ([2003](#)), where c_{min} = lowest concentration in the gradient, P_0 = pellet size in the control well, d = 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.

A second method was used to calculate inhibitory concentrations o f toxicants to the MARA species. For each toxicant/sample the growth values provided by the MARA software were exported to Microsoft Excel and the Excel macro, REGTOX (http://eric.vindimian.9online.fr/en_index.html), which models the dose–response relationship with the non-linear Hill equation, was used to calculate IC_{20} (20% Inhibitory concentration) and IC_{50} (50% Inhibitory concentration) for each MARA species. The 50% yeast growth inhibitory concentrations (IC_{50}s) were also calculated for each yeast species using REGTOX. A regression was then performed between the MTC values calculated by the MARA software and the IC_{20} and IC_{50} values from REGTOX for all samples.

Results

MARA results

Figure [1](#) is a summary of the MTCs, IC_{20}s and IC_{50}s for each MARA species and the respective means of these values for all MARA species for each of the samples used in this study. It can be seen that for each sample there is a wide range of sensitivities between the MARA species and the most sensitive species are different for different samples. Of particular interest to this study is MARA species 11, which is the yeast *Pichia anomalia*. It is clearly the most sensitive species to the fungicides maneb and captan (Fig. [1](#)b), with the lowest maneb concentration of 1.56 mg/l causing 100% growth inhibition in this species. On the other hand, it is the most tolerant species to potassium dichromate (Fig. [1](#)a) and among the more tolerant species to the industrial effluent (Fig. [1](#)c). This yeast sensitivies to 3,5 dichlorophenol (Fig. [1](#)a) and the soil leachate (Fig. [1](#)c) are comparable to those of many other MARA species for these samples. Figure [1](#) also shows that the mean MTC and IC_{20} values for each sample are in most cases close to each other and represent the concentration of toxicant that inhibits the growth of the more sensitive species, while the mean IC_{50} is the concentration that inhibits the growth of most of the MARA species. Across all samples, there is a good correlation between the MTC calculated using the MARA
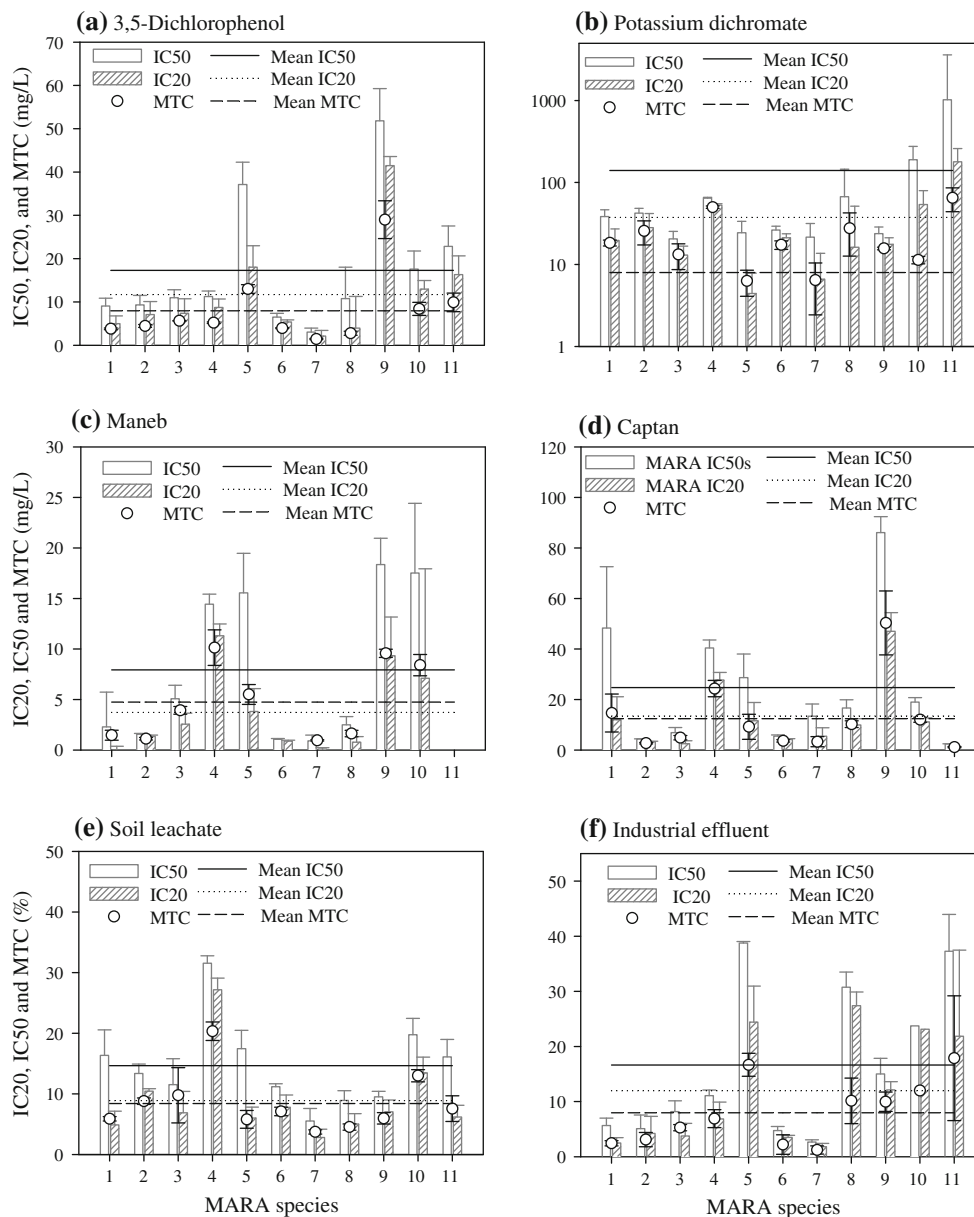
Fig. 1 Summary of MARA MTCs (calculated by MARA software), $IC_{20}s$ and $IC_{50}s$ (calculated using REGTOX) for all samples (*Error bar* 95% confidence limits for IC_{20} and IC_{50} and ± 1 SD for MTC)

software and the IC_{20} and IC_{50} values calculated from the same data using REGTOX (Fig. 2).

Comparing the sensitivities of MARA species with those of nine yeast species

The yeast growth inhibition bioassay could be considered as similar to the MARA test, because it involved the growth of yeast species for 24 h. The MARA species IC_{50} values were, thus, compared with the yeast growth $IC_{50}s$ for the different toxicants used in the present study (Fig. 3).

For potassium dichromate and captan (Fig. 3 a, b) the mean growth $IC_{50}s$ from both sets of organisms fell within the same range. However, in the case of potassium dichromate, most of the MARA species had IC_{50} values well below the mean IC_{50} and only two species (no. 10, 11) were largely responsible for raising the mean IC_{50} value. The mean growth IC_{50} for yeast species were respectively 32 and 44% higher than corresponding mean IC_{50} values of MARA species in the cases of maneb and the industrial effluent (Fig. 3 c, d). On the other hand, the yeast species had a lower mean growth IC_{50} (58% less) value than
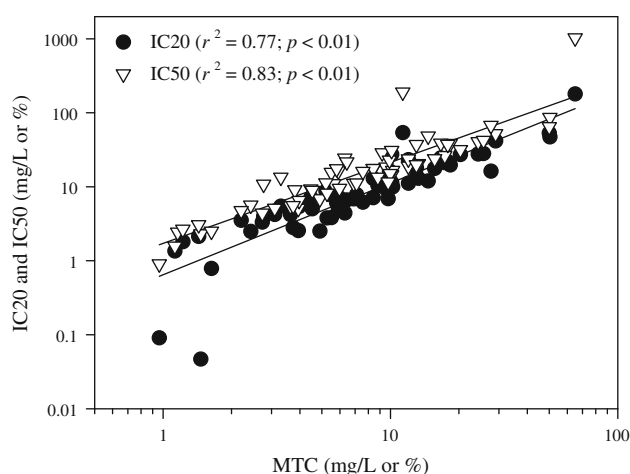
Fig. 2 Regression of MTC values against the IC_{20} and IC_{50} values for all samples combined

MARA species for the soil leachate (Fig. 3e). Generally, there was a wider IC_{50} range among the MARA species to each toxicant than among the yeast species. Overall, therefore, the mean MARA and yeast growth IC_{50}s were comparable, but the MARA organisms exhibited more diverse sensitivities, which is not surprising given that they are phylogenetically more diverse. The single yeast species in the MARA plate (*P. anomalia*) was more sensitive than the set of nine yeast species to the fungicides (Fig. 3b and c), but it was less sensitive than the other yeast species to potassium dichromate (Fig. 3a).

Discussion

MARA is a recently developed multispecies test with many desirable qualities such as cost-effectiveness, simplicity and ease of use (Gabrielson et al. 2003; Wadhia et al. 2007). However, for this test to be incorporated into the regulatory framework there is need to obtain more data with it in order to establish the test performance (Greenwood et al. 2007).

MARA results for 3,5-dichlorophenol, potassium dichromate, captan, maneb and two environmental samples are presented in the present study. MTC was introduced by Gabrielson et al. (2003) as a new method for expressing the degree of toxicity, therefore, MARA results are usually presented as MTC values (Gabrielson et al. 2003; Wadhia et al. 2007), but more information, such as the complete dose–response curves, as well as IC_{20} and IC_{50}, can also be obtained from the MARA growth data. The MTC values reported here for 3,5-dichlorophenol were comparable to published MARA species MTCs for this toxicant (Wadhia et al. 2007). The sensitivities of the different MARA species, varied widely for each toxicant and from one sample to the other in the present study. This agrees with previous

studies and has been attributed to the wide genetic diversity of the selected MARA species (Gabrielson et al. 2003). This is a great advantage for the MARA test as it increases the likelihood of detecting toxic effects from a wide range of contaminants. A good correlation has been established in the present research between the MARA MTCs (calculated using the MARA software) and IC_{20} and IC_{50} values (calculated by REGTOX) from the same data sets. MARA MTC values have previously been equated to IC_{50}s and were, thus, compared with IC_{50} values from other bioassays (Gabrielson et al. 2003; Wadhia et al. 2007), but from the present results they were generally more comparable to IC_{20} values. The mean MTC and IC_{20} values and their respective confidence limits represented the toxicant concentration ranges that affected the more sensitive micro-organisms, while the mean IC_{50} corresponded to the concentration that was toxic to most of the test organisms. This information is very relevant for risk assessment.

For comparison of MARA results with other bioassays, MARA IC_{50} values were used. It could be seen that the IC_{50}s for individual MARA species were in many cases lower than yeast growth IC_{50}s although the mean IC_{50}s from both tests were mostly comparable with each other. In addition, MARA IC_{50}s for the more sensitive species were comparable to the corresponding literature IC_{50} (or LC_{50}) values for *D. magna*, *S. capricornutum* and Microtox bioassays, although the mean MARA IC_{50} values were higher than those from these bioassays (Table 1). Considerable differences in sensitivity among micro-organisms is known to exist (DeLorenzo et al. 2001). Because the most sensitive MARA species is different for different toxicants, the MARA test is likely to detect many more toxicants than single species tests.

The yeast was the most tolerant MARA species to potassium dichromate and seven of the nine yeast species also had higher growth IC_{50} values than most MARA species for this chemical (Fig. 3a) agreeing with other reports in literature that yeasts are especially tolerant to heavy metals (Falih 1998; Fai and Grant 2009a). However, all yeast species in the present study were as susceptible as the other MARA species to the soil leachate (Fig. 3e), which was a composite sample containing metals. On the other hand, the MARA yeast species (*Pichia anomalia*) was the most sensitive MARA species to both fungicides used in this study. This is expected, since fungicides are meant to target fungi. In addition, both fungicides, maneb and captan, have the same mode of action. They are both non-specific thiol reactant fungicides that work by inhibiting several enzymes involved in respiration (Leroux 2003). *Pichia anomalia* was also more sensitive to both fungicides compared to the nine other yeast species in the present study. However, the fungicides also seriously inhibited the growth of at least six other bacterial species
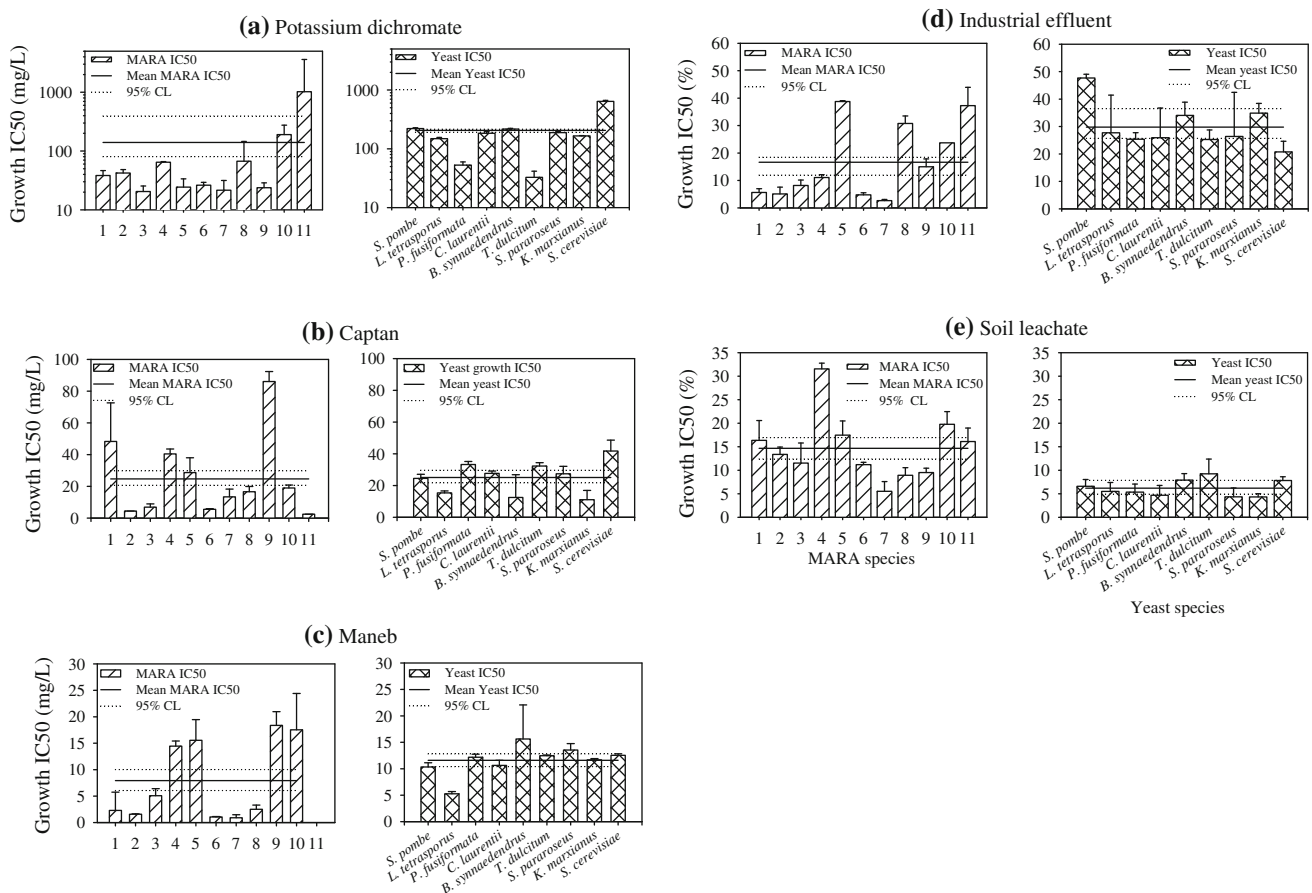
Fig. 3 Comparing MARA species IC$_{50}$s with yeast species growth IC$_{50}$s (*Error bars* 95% confidence limits, CL = confidence limits)

Table 1 Comparing MARA IC$_{50}$ values with literature values from other bioassays

Toxicant	MARA IC$_{50}$ (mg/l)		*Daphnia magna* 24 h LC$_{50}$ (mg/l)	*Selenastrum capricornutum* 72 h IC$_{50}$ (mg/l)	Microtox IC$_{50}$ (mg/l)
	Minimum	Mean			
3,5-Dichlorophenol	3.1	14.6	2.1[a]	0.9–3.6[b]	4.1(Reteuna et al. 1989)
K$_2$Cr$_2$O$_7$	21.6	140.1	0.3–2.4[b]	0.9–1.8[b]	15(Reteuna et al. 1989)
Captan	2.43	24.7	1.3[a]	2.4[b]	–
Maneb	< 1.6	7.9	0.8–1.3[b]	0.014[b]	–

[a] http://www.pesticideinfo.org/Search_Ecotoxicity.jsp

[b] http://cfpub.epa.gov/ecotox/quick_query.htm

demonstrating the effects of fungicides to non-target organisms. A similar picture is seen for all other tested samples, indicating the potential of MARA for assessing the effects of fungicides and other toxicants on non-target organisms.

One novelty of MARA is the concept of toxicity fingerprinting and pattern recognition based on the comparison of the toxic fingerprint from a tested compound to a database consisting of fingerprints from a large number of previously tested compounds with known toxic properties

(Gabrielson et al. 2003). The pattern of toxicity of both fungicides to MARA species seem to be similar (Fig. 1b) with MARA species 4, 5 and 9 being more tolerant than the others, while 2, 3, 6, 7, 8 and 11 were very sensitive to both fungicides. This could indicate the beginning of a toxic fingerprint for organic fungicides, but more work needs to be done. For example, in case of captan there was some diversity in response between the yeast spp. This indicates that, depending on the compound, a species can be more or less sensitive. To check whether *P. anomalia* is a good

representative of sensitive spp for fungicides in general, a further array of fungicides (with different modes of action) needs to be tested. The industrial effluent was from a large chemical company, which manufactures a wide range of specialised organic chemicals, such as crop protection products, petrochemicals and biocides. The sample used in this research was not analysed, but because all yeast species, including the MARA yeast species, were not particularly sensitive to it, the presence of fungicides in this sample could be ruled out. This is one way MARA toxicity fingerprints can increase the chances of success in toxicant identification in effect-directed analysis studies where the chemical composition of samples is largely unknown.

In summary, therefore, we find the MARA test to be very advantageous for several reasons. Firstly, its protocol is simple to follow. Secondly, the test is neither labour-intensive nor time-consuming. Thirdly, and most importantly, it is sensitive to a broad range of chemicals and environmental samples and is cost-effective, especially considering the fact that results are simultaneously obtained for 11 species of organisms. However, the comparison of MARA MTCs with toxicity end points from other bioassays should be done with caution as shown in the present study that they are more comparable to IC_{20}s than IC_{50}s. In addition, there is no primary producer among the MARA micro-organisms implying that for a more complete risk assessment of pesticides, it would be useful to also do an algal bioassay in order to detect effects of herbicides. It is concluded that the MARA test has a great potential for ecotoxicological testing of chemicals and environmental samples and would be very valuable for use by regulatory bodies for assessing the effects of pollutants.

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