MICROBIAL ASSAY FOR RISK ASSESSMENT – A NEW INNOVATIVE DEVELOPMENT FOR TOXICITY TESTING OF CHEMICALS AND ENVIRONMENTAL SAMPLES

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ABSTRACT

Microbial Assay for Risk Assessment (MARA), an array of eleven genetically diverse microorganisms, freeze-dried in a micro-titre plate, was used for the evaluation of toxicity of various environmental samples ranging from complex industrial effluents to potable water samples. The array was exposed to a concentration gradient of the test sample in the presence of a growth medium. Growth of the microorganisms in the array at each sample dilution was measured by recording the reduction of the redox dye tetrazolium red.

After incubation the plates were scanned utilising a flat bed scanner. The image produced from this was analysed using purpose-built software to provide a unique fingerprint of the sample expressed in terms of sensitivity of the constituent ten prokaryotic and one eukaryotic (yeast) species. Sample toxicity assessment using MARA is conveyed in terms of a microbial toxic concentration for each microbial species and the overall assay mean. These values generated by the software are determinations equivalent to EC_{50} values.

The system can also be used for initial screening of samples of potentially low (or zero) toxicity. In this instance, the percentage growth inhibition of each of the eleven microorganism upon exposure to the neat sample is ascertained by comparison with the test control.

The results of the testing performed with the MARA system were compared with toxicity assessment made using conventional ecotoxicity methods e.g. Microtox®. The inference of the evaluation implemented concluded that MARA is a useful tool for ecotoxicity testing.

KEY WORDS

Bioassays; Biological assessment; Ecotoxicity; Effluent; Leachates; Microbial assay; Soil extracts; Waters

INTRODUCTION

ECOTOXICITY TESTING

The use of chemical analysis has been the predominant means of assessing the potential impact of toxicants to the environment. A comparison of the toxicant composition of environmental samples with established guideline values has allowed the determination of the acceptable levels of contamination [1]. However, a drawback of this approach has been the definitive inference of toxicity to the biota in the ecosystem in view of the predictive element and the complexity concerning the evaluation of bioavailability [2].

Tests or bioassays, initially developed to characterise the toxicity of chemicals, have proved to be a valuable asset for the toxicity evaluation of environmental samples [3, 4]. The utilisation of bioassays both in the regulatory and non-regulatory framework is now evident worldwide [5, 6]. Protection of ecosystems has been largely implemented with the use of single trophic species, including invertebrates and algae. The potential use of microbial tests has been recognised with the evident proposals submitted by regulatory and standardization organizations [7]. A number of tests employing bacterial species are commercially available. These have key benefits but are essentially disadvantaged in that they are based on a single species.

MICROBIAL ASSAY FOR RISK ASSESSMENT (MARA)

An innovative development of significant potential is the MARA (Microbial Assay for Risk Assessment). It is a multi-species assay which allows measurement of toxic effects of chemicals and

environmental samples. The test uses a selection of taxonomically diverse range of microbial species lyophilised in a microplate. Ten prokaryotic species and a eukaryote (yeast) constitute the biological indicators of toxicity assessment.
The growth of the organisms exposed to a dilution series of the test sample is determined with the reduction of tetrazolium red (TZR). A scanned image of the microplate obtained using a flatbed scanner is analysed using purpose-built software.

MARA EVALUATION

Testing was performed using the Microbial Assay for Risk Assessment (MARA) to assess the toxicity of an extensive range of environmental samples. Samples tested included raw and treated waters, effluents, sewage sludges and soil leachates. The performance of the assay was evaluated with comparative assessment involving pertinent tests routinely utilised for toxicity determination. This included respiration and nitrification inhibition tests employed to protect STWs from potential throughput of toxic discharge. The evaluation was implemented to assess the application of MARA in testing the toxicity of a spectrum of aquatic and terrestrial environmental samples.

ASSAY PROTOCOL

SAMPLE PREPARATION

Where necessary, samples were centrifuged (at 3000rpm for 15 min) to remove particulate matter. Each sample was filtered by means of a 10ml syringe through a 0.22 or 0.45 micron filter into a clean universal bottle to remove the natural flora. A total of 10ml sample was prepared. Phytone peptone (0.4g) was added to the sample. After dissolution of the solid medium, 0.2ml tetrazolium red (TZR, 1% w/v solution) was added. Samples were analysed immediately after preparation.

PLATE PREPARATION PRIOR TO SAMPLE TESTING

All plates were allowed to equilibrate (for 2 hours) to room temperature after removal from their protective packaging. A volume of 150µl of medium was added to each well containing the microorganisms (Rows H, columns 1 to 11 – see Figure 1) and to the last well in row H in column 12. The latter contained the freeze-drying base only and was used as a contamination control since it was treated with sample in the same way as the microorganisms in the plate. Any biomass observed in wells in column 12 was attributed to contamination since all samples were filter sterilised. After the addition of growth medium, all plates were incubated for 4 hours at 30ºC in a sealed plastic container containing moist tissue paper to ensure that constant humidity was maintained.

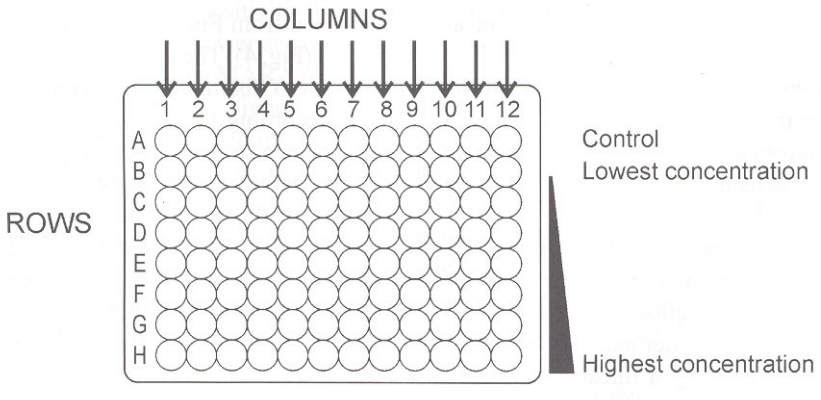

Figure 1 MARA 96-well microplate

SAMPLE DILUTION SERIES

After incubation the plates was removed from the incubator and 150µl of medium containing TZR (0.01% w/v) was added to each well in Rows A to F. A volume of 150µl of sample was added to the wells in Row G. Using a 12 channel pipette 50µl of sample was transferred from each well in Row G to the corresponding well in Row F. The contents of the wells in Row F were mixed by filling and discharging the 12 channel pipette into the wells. After mixing 50µl of the contents of the wells in Row F were transferred to the wells in Row E. This procedure was repeated to Row B. The contents of the wells in Row B were mixed as before and 50µl were discarded from each well in the row. In this manner a concentration gradient (in 3 times steps) was obtained from Row G (highest concentration) to Row B (lowest concentration). The concentration gradient of the test environmental sample ranged from 100% (in Row G) to 0.4% (in Row B). No sample was added to the wells in Row A. This

constituted the negative control. Using the above procedure a constant reaction volume of 100µl was obtained in all the wells in Rows A to G.

INOCULATION AND INCUBATION

Finally, 15µl was transferred, after mixing, from the wells in Row H to the corresponding wells in Row A, using a 12 channel pipette; this was repeated through Rows B to G. In this manner all the wells in each column were inoculated with the same volume of microorganism. Each plate was then placed in a plastic container as before and incubated at 30ºC for 18 hours.

TEST MEASUREMENTS

After incubation the plates were removed from the incubator. Any condensation on the base of the plates was carefully removed using tissue paper, care being taken not to shake the plates. Growth in each plate (after removal of the lid) was recorded by means of a scanner (HP Scanjet 7400c) using transmitted light and a resolution of 100. The scans of plate images were stored and subsequently analysed using a specially designed software package to determine, wherever applicable, a microbial toxic concentration.

MARA TOXICITY DETERMINATION

Evaluation of the data produced with the MARA is assessed by examining the growth inhibitory effect on the pellet formation at each sample dilution. The assessment can be made for each individual species or by looking at the mean software-computed value of all the constituent species. Using the latter the toxicity of a sample can be expressed in reference to a threshold value or interval assigned for toxicity classification. For example, an undiluted sample exhibiting an overall inhibitory effect (across 11 species) of <20% is considered to non-toxic. This is an effective means of attaining a rapid inference of the results.

In order to provide a comprehensive and optimal assessment utilising the significant feature of the MARA as a multi-species test, a determination referred to as the *Microbial Toxic Concentration* (MTC) is computed. The MTC value is determined as follows:

$$MTC = c_{min} \times d^{P_{tot}/P_o) - 1}$$

c_{min} = lowest concentration in the gradient
P_o = pellet size in the control
d = dilution factor
P_{tot} = sum of all pellet sizes across the concentration gradient

MTC values for the MARA are generated for each species and as a single value for the assay as a whole. The specific species-related MTC values provide a *MARA toxic fingerprint* uniquely characteristic of the test substance and potentially indicative of the mode of toxic action.

The MTC is a means of computing a value equivalent to the EC_{50} determination. The computation uses all available points above and below the growth curve in the zero to hundred percent inhibition range.

Note 1 – To facilitate comparative assessment, MARA MTC is referred to as MARA EC_{50} in this paper.

Note 2 – A vast number of environmental samples were tested using the MARA but for the purpose of providing examples a selected few are mentioned here.

TEST RESULTS AND EVALUATION

RAW WATERS

Samples of borehole water and river water were tested using the MARA. The results presented in Figure 2 exhibited inhibition of pellet growth of the most sensitive species but the overall assessment generated with the software provided the inference that the samples were non-toxic.

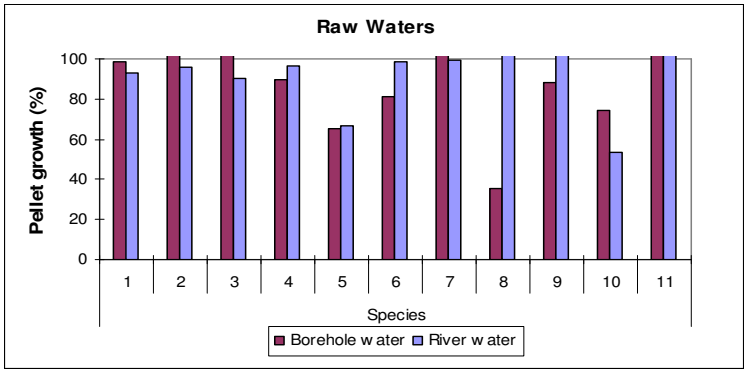

Figure 2 Mean pellet growth of replicate(x3) wells compared to test control of MARA species with test performed using 'raw' waters

The samples were also tested using Microtox® and the results indicated inhibition values below the limit of detection of this test.

EFFLUENTS

A number of different effluents were tested using the MARA and a broad range of toxicity values were attained. Histograms of EC_{50} values for individual MARA species are given in Figures 3 and 4. The EC_{50} mean value of all species and that of the most sensitive species are shown as line graphs.

SAMPLE 060005677

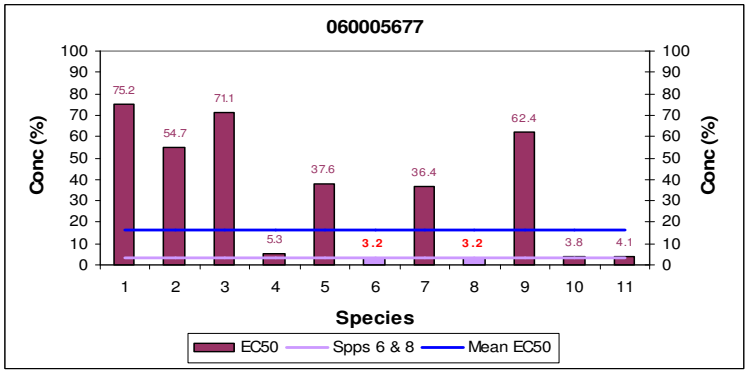

Figure 3 MARA EC_{50} determinations

For sample 060005677 the MARA mean EC_{50} value obtained was 16.5%. Using Microtox® an EC_{50} value of 42.1% was recorded; thus for this sample the MARA exhibited better sensitivity than Microtox®. Chemical analysis and additional ecotoxicity tests results for sample 060005677 are given in Table1. Respiration inhibition test performed using sample 060005677 resulted in a x1.54 dilution (65% sample concentration) giving 45.7% inhibition. A sample concentration of 17.5% (x5.7 dilution) was required to give an EC_{50} value with the nitrification inhibition test. Thus the MARA shows potential scope for utilisation for the protection of STWs against toxic effluent discharge.

SAMPLE 060001181

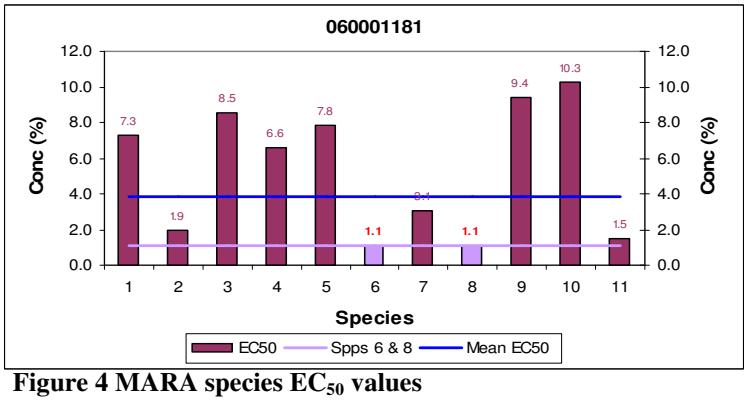

Figure 4 MARA species EC_{50} values

The sample tested 060001181 was an industrial effluent of potential concern to a sewage treatment work and required regular monitoring. The source of the effluent was a chemical manufacturing plant. Mean EC_{50} values obtained for the MARA species ranged from 1.1% to 10.3%; the sample mean EC_{50} value was determined to be 3.9%.
The results of the testing with other ecotoxicity tests gave the following EC_{50} values:

Respiration inhibition – 8.6%

Nitrification inhibition test – 0.4%

The results showed that the MARA test for this sample was more sensitive than Respiration inhibition test. And the latter is routinely employed to assess the toxicity of this effluent because of its ecological or operational relevance.

SEWAGE SLUDGE

Mean EC_{50} value for the sample was determined to be 35%. Inhibition values with MARA for the neat (100%) and 33.3% sample concentration ranged from 0 to 100% for the 11 species. Thus, although the sample had an intrinsic low toxicity, the most sensitive MARA species detected the presence of toxic components.

SAMPLE 060007866

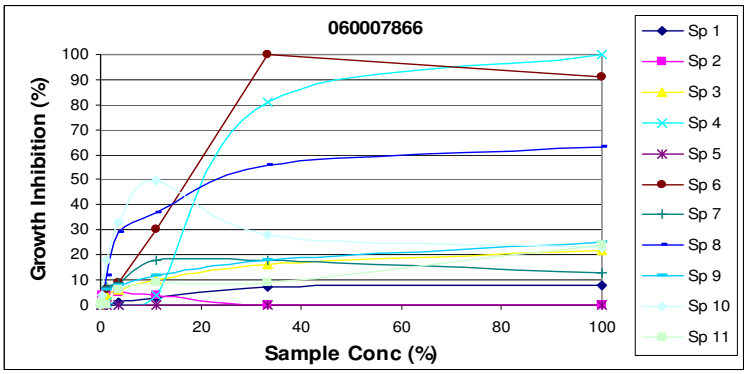

Figure 5 Growth inhibition plots for MARA species

SEWAGE ASH

An aqueous leachate (10:1) of a sewage ash from an incineration plant was tested using the MARA. The leachate exhibited low toxicity for most species and the overall mean inhibition for all species was determined to be 30%. Any potentially toxic elements in the ash were at low concentrations and/or not leachable. Testing performed using Microtox® indicated that the leachate toxicity was below the limit of test detection.

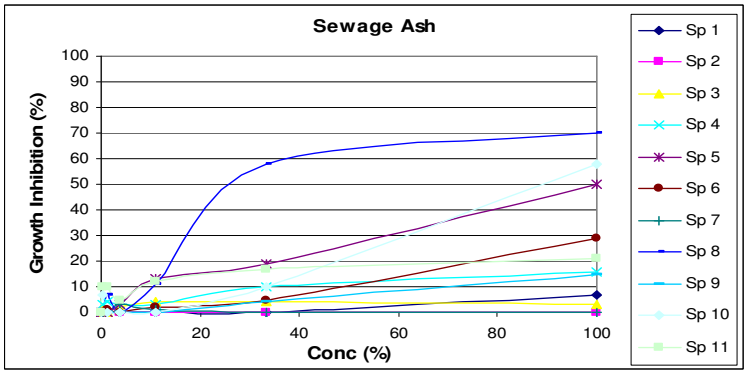

Figure 6 Growth inhibition of MARA species exposed to sewage ash leachate

SOIL LEACHATES

Soil samples were tested using 10:1 aqueous leachate preparations. MARA results showed that low leachable levels of toxicants, confirmed with chemical analysis, did not result in significant (<20%) toxicity. Some species were found to be sensitive to the soil leachates (see Figure 7).
Further comparison with leachates of reference (LUFA) soils tested using MARA and Microtox® confirmed that detectable toxicity attained with the soil samples was attributable to toxicant components in the soil matrix.

SAMPLE 060004471 – SOIL LEACHATE

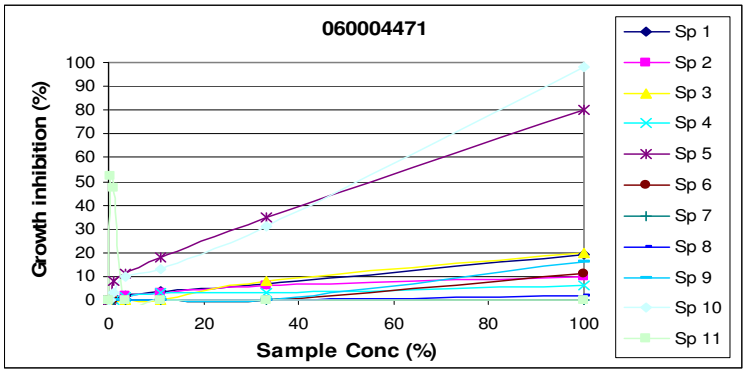

Figure 7 Effect on MARA species exposed to soil leachate

MARA PERFORMANCE EVALUATION

In addition to the testing of environmental samples, extensive assessment of the MARA to investigate sensitivity to metals and organic toxicants and confounding variables was performed. In addition a measure of the MARA's variability was determined with repeatibilty and reproducibility trials.

CONCLUSIONS

1. The concept and design of the Microbial Assay for Risk Assessment (MARA) potentially offers a significant improvement on existing ecotoxicity tests. The use of multiple species in the MARA test is ecotoxicologically of substantial importance. As toxicity is both chemical and species specific, the use of a battery of species is critical.

2. A significant asset of the MARA is that toxicity assessment is ascertained with reference to the most sensitive species, and in addition the overall toxicity to a battery of phylogenetic diverse strains. This provides a valuable means of attaining unique toxic fingerprints of different chemicals.

3. Performance results of the MARA test evaluations indicate that the precision and consistency exhibited by the test allows an accurate assessment. The MARA test was found to be as sensitive as some other established ecotoxicity tests with reference to specific toxicants tested in this trial. The assessment although based on a limited number of comparisons both in terms of tests and toxicants, shows scope for routine employment of the assay. The MARA is capable of detecting inorganic and organic toxicants.

4. The results of the testing performed using the MARA indicates that it is suitable for assessing the toxicity of different types of environmental samples. The assay can be potentially used for assessment of soil samples, sewage sludges/ashes and effluents.

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TABLES

TABLE 1. CHEMICAL ANALYSIS, RESPIRATION AND NITRIFICATION INHIBITION TESTS RESULTS FOR SAMPLE 060005677

Determinand		**Unit**
1,2-Dibromoethane	167	µg/l
Ammoniacal nitrogen	29.6	mg/l N
Arsenic (total)	2.38	µg/l
BOD (5 day)	547	mg/l O
Cadmium (total)	<0.005	mg/l
Chromium (total)	<0.01	mg/l
COD (total)	1820	mg/l O
Copper (total)	<0.01	mg/l
Iron (total)	0.357	mg/l
Lead (total)	<0.03	mg/l
Mercury (total)	<0.05	µg/l
Nickel (total)	0.0134	mg/l
Nitrification inhibition	84.6	%
Nitrification inhibition dilution	2.20	
Nitrification inhibition dilutions to EC_{50}	5.70	
Respiration inhibition dilution	1.54	
Respiration Inhibition Result	45.7	% inh
Suspended solids (neutralised)	153	mg/l
Zinc (total)	<0.02	mg/l

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