

3.13. RAD54-GFP

Dr. Richard M. Walmsley^a, Patrick Keenan^a, Dr. Andrew W. Knight^b and Dr. Jacqueline Schmuckli.^c

^a Department of Biomolecular Sciences

^b Department of Instrumentation and Analytical Science

University of Manchester Institute of Science and Technology PO Box 88, Manchester, M60 1QD, U.K.

Tel: ++ 44 (0)161 200 4174/6

Fax: ++ 44 (0)161 236 0409

E-mail: p.keenan@stud.umist.ac.uk

walmsley@umist.ac.uk

a.knight@umist.ac.uk

^c Institut für Allgemeine Mikrobiologie, Baltzer-Strasse 4, CH-3012 Bern, Switzerland

Tel ++ 41 (0)31 631 46 57

FAX ++ 41 (0)31 631 46 84

E-mail: schmuckli@imb.unibe.ch

Introduction

Justification of the test:

The UMIST RAD54-GFP test utilises yeast, a eukaryotic organism, as the biological component of the biosensor. The biological core of the biosensor has already been developed beyond proof of principal, in the project "EMFID" – Environmental Monitoring by Fluorescence Induction and Detection. The existing biosensor is the product of a multidisciplinary collaboration between biologists and instrumentation scientists, fostered by the EC Environment and Climate Programme (Framework 4, DGXII) and has resulted in a number of relevant publications (Walmsley *et al.*, 1997; Billinton *et al.*, 1998; Knight *et al.*, 1999a & b; Knight *et al.*, 2000; Afanassiev *et al.*, 1999)

By using yeast, the UMIST biosensor overcomes a number of problems associated with non-eukaryotic systems. Parry recently reviewed the use of tests in yeast and fungi in the detection and evaluation of carcinogens for the International Agency for Research on Cancer (IARC Scientific Publications, 1999 No. 146 p471-485). He noted the physical robustness of these organisms, in comparison to bacteria, with pH tolerance between 3 and 9, survival from freezing to over 40°C and wide tolerance of osmolarity / ionic strength. Furthermore, the clinical utility of the majority of highly bactericidal compounds (e.g. antibiotics) is based on their selectivity against prokaryotes (and prokaryotic assay systems!) but as such they do not affect yeasts.

Yeasts are an attractive model because they are eukaryotes like mammals. They have eukaryotic architecture with internal organelles and similar chromosome structure and DNA repair processes. By incorporating a human cytochrome P450 enzyme into the yeast, *in vivo*

metabolic activation of promutagens is obtained, replicating more accurately, the human response.

Furthermore, there is a valuable literature describing the utility of yeasts. This has arisen because of early efforts produce a yeast version of the bacterial reverse mutation (Ames) test. As early as 1984, Mehta and Von Borstel had made a study of a highly carcinogenic group of (N-nitroso) compounds that were genetically inactive in Salmonella (Ames) mutagenesis tests but produced cancer in rats. All could induce mutations in yeast. In 1994, Carls and Shiestl reported a second type of yeast mutation assay ('Del') and the genotoxicity of a still wider range of compounds to yeast. These other yeast tests share one major drawback with the bacterial reverse mutation tests: they require time-consuming plating out, and competent microbiologists to carry them out. The new UMIST RAD54-GFP test involves no plating, and can be automated.

Description of the System

The test incorporates a genetically modified yeast (*Saccharomyces cerevisiae*) as the biological component of a biosensor. On exposure of the yeast to a genotoxin, green fluorescent protein (GFP) is produced which, under excitation by blue light (wavelength 490nm), emits green fluorescence that can be measured using a variety of conventional photodetectors (Billinton *et al.*, 1998). Cytotoxicity can be assessed by measuring the rate of change in cell density / optical density (at 600nm) of the yeast culture, or the density achieved by a fixed time point after induction. The fluorescence is normalised by dividing the amount of fluorescence by optical density, to give essentially "fluorescence per cell".

Principle of the assay

The transformed yeasts contain a plasmid incorporating the promoter of the RAD54 gene that is an inducible constituent of the DNA repair system in yeast. It responds to all agents known to be mutagenic to yeast. The promoter is fused to a gene encoding GFP. Upon exposure to a genotoxin, the DNA repair system and its associated genes, including RAD54, are upregulated (Walmsley *et al.*, 1997). As a result, the production of GFP is also upregulated. The amount of GFP accumulated reflects the genotoxic effect of the substance *i.e.* the more damage caused, the greater the activation of the DNA repair system, the greater the amount of GFP produced. This GFP can then be non-invasively quantified as described.

The strain incorporating the gene for a human cytochrome P4501A2 allows the metabolic activation of promutagens which then cause expression of GFP as described above.

Host/organism:

The host is yeast strain FF18984 (supplied by Francis Fabre). The strain is transformed with either a single plasmid or, when metabolic activation is required, two separate plasmids. The plasmids are; pWDH444 (Walmsley *et al.*, 1997) for the normal strain used with toxins that do not require metabolic activation, and a dual plasmid strain incorporating the same pWDH444 reporter construct plus a construct containing a P450 gene (pCS513; supplied by Christian Sengstag). A wild type, unmodified strain was also used to correct for the small cellular auto-fluorescence component of the measured fluorescence signal if necessary.

Molecular target

The molecular target is DNA. The reporter responds directly to DNA damage. The test can also detect cytotoxicity, a result of more general macro-molecular damage.

Mechanism of genotoxicity

The mechanism of genotoxicity is the activation or up-regulation of the DNA repair system in response to DNA damage however caused, whether by chemical genotoxins or ionizing radiation.

Point measurements or kinetics

With the system it is possible to carry out both point and continuous / kinetic measurements. Kinetic measurements (not carried out at the Technotox Workshop) can be performed by repeated measurements of the microtitre plate housed within an incubating / shaking plate reader. Alternatively this could be more accurately performed by using an in-house designed and manufactured dual channel, continuous flow, GFP monitoring instrument which follows GFP expression and cell growth in both a control and sample culture.

Format of the test:

For the Technotox Workshop, the 96-well microtitre plate format was used, however cuvette, tube and flask formats are also used in other lab-based assays. Following a pipetting protocol, at the initiation of the test, each well contained 75 µl of test sample at the appropriate dilution, (produced by on-plate serial dilution), to which 75 µl of yeast cell culture was added, at an optical density of 0.2, in double strength media. The plates were shaken and incubated overnight, after which fluorescence and absorbance was read.

Apparatus needed to perform the test:

The major pieces of equipment required for this test were the BMG Fluostar Galaxy (BMG LabTechnologies, Aylesbury, UK) which is a combined fluorimeter (for both polarised and non-polarised light), luminometer, spectrophotometer, and a shaking incubator. Additionally, a new portable fluorimeter, designed and built within the Department of Instrumentation and Analytical Science at UMIST, was bench-tested for the first time.

Exposure time:

Upon exposure of the yeast to a genotoxin, a response can be clearly seen after 4 hours. During the workshop readings were taken after both 4 hours and 17 hours (overnight). At 4 hours a qualitative assessment of genotoxicity can be established, basically a yes or no result. At 17 hours, after the cells have reached their peak induction, a more reliable qualitative assessment of genotoxicity could be made. GFP is a very chemically and photochemically stable molecule, and after expression remains intact in the yeast cell for long periods, up to several months. Interestingly the "transformed + P450" strain showed the fastest response with a clear dose response curve evident after just 4 hours - see later and figures 9 and 10.

Dilution of the sample in the test

Each sample tested was subjected to ten, two-fold serial dilutions, either with water or 2% DMSO (2% final concentration for samples supplied in 2% DMSO).

pH and salts requirements

All pH and salt requirements are met by the 'F1' media (Walmsley *et al.*, 1997) used throughout this workshop. Furthermore, due to the physical robustness and wide pH tolerance of yeast (see previous section on the justification of the test), the pH and ionic strength properties of the sample are not expected to adversely affect the system.

Frequency of false positives/negatives

Trivial false positives can occur with highly auto-fluorescent compounds. However, due to the sensitivity of the assay these can be removed by serial dilutions and the subtraction of blank values. The presence of some auto-fluorescent compounds can also be corrected for in the recorded fluorescence, by exploiting differences in fluorescence polarisation properties between the autofluorescent species and GFP, which has a high fluorescence anisotropy (Knight *et al.* 1999b and 2000). Highly coloured samples can also lead to false negatives if they absorb at the wavelength of interest, but this problem is also eliminated by serial dilution and comparison to the blank.

Since eukaryotic cells are used, of the many compounds tested so far with the biosensor, no problems with false negatives or positives have been observed. Some of these have included compounds that have provided false positives or negatives with the Ames assay.

Availability as a Test kit

This test is currently being developed for commercial use.

Used for screening/pre-screening/regulatory purposes

The test is currently designed for a pre-screening role. It may be possible in the future to use the test in a regulatory role, although this will require regulatory approval.

Solvent requirements

Solvents are only required where they necessary to solubilise chemicals. However DMSO also acts to increase the permeability of the cell wall which enhances the sensitivity of the test. This was particularly apparent when comparing the results of 4NQO as a standard (prepared and diluted in the test in DMSO) and as a spike to the river sample (diluted in water). In the latter case the NQO signal was inconclusive, suggesting a solvent matrix effect.

Analysis of all categories of compounds

The test also measures cytotoxicity via cell growth. None of the compounds tested to date have inhibited the growth of cells to the extent that the test becomes no longer valid. Additionally, yeast, in common with many organisms, has genes whose products mitigate against heavy metal poisoning.

Maximum number of samples/working days

For the Technotox Workshop, all eleven samples, including dilution ranges, were processed in one day. In addition, a twelfth sample (MMS) was tested. MMS is the standard carcinogen used among the research group in UMIST. Replicates were prepared the following day. However, the protocol would readily lend itself to robotic automation with high sample throughput.

Metabolic activator

Two strains were used, one of which had *in vivo* metabolic activation. The metabolic activator used in this case was P450. Human cytochrome P450 1A2 was co-expressed with its recycling oxidoreductase in the yeast system used (*manuscript in preparation*).

Technology phase

The yeast test is in the late research and development stage. Instrumentation prototypes have been developed and a systematic validation exercise of the instrumentation and protocols commences summer 2000.

Main potential applications domains

It is envisaged that the assay will be used mostly in the fields of pharmaceutical screening along with continuous and remote environmental monitoring.

Environmental relevance

One of the on-going areas of research at UMIST is the optimisation of the biosensor for use with environmental samples. The research is in part funded by EC DGXII Environment and Climate programme. Through the UMIST - based Biotechnology Transfer Initiative we are working with SME's in North West England to assure a user-friendly format for the 'problem owners'.

Results

Table 1 summarises the findings for each of the samples provided, as listed above. For each sample an indication of a positive genotoxic result is given, along with the concentration tried which gave the peak induction, the lowest detectable concentration and the lowest ineffective concentration. Also listed is an indication of a positive cytotoxic response, and the corresponding lowest ineffective dilution for cytotoxicity. Finally an indication of genotoxicity as measured by a newly developed portable instrument is given.

RAD 54 - GFP - YEAST TEST

| Sample | | Genotoxic | Peak Induction | Lowest Detectable | Lowest Ineffective | Cytotoxic | Lowest Ineffective | (Portable Device) Genotoxic |
|---------|------------------------|-----------|----------------------|----------------------|----------------------|--------------|----------------------|-----------------------------|
| Number | ID | + / - | Conc. / ng/ml | Conc. / ng/ml | Conc. / ng/ml | + / - | Conc. / ng/ml | + / - |
| S1 | 4-NQO 5 µg/ml | + | 500 | 3.9 | 2.0 | + | 125 | (+) |
| S2 | 4-NQO 0.1 µg/ml | (+) | 5 | 1.3 | 0.6 | - | - | (+) |
| S3 | MNNG 400 µg/ml | + | 5000 | 156 | 78 | + | 625 | + |
| S4 | 2-AA 4 µg/ml | (+) | 50 | 3.1 | 1.6 | + | 50 | + |
| S5 | 2-AA 200 µg/ml | + | 20 | 4.9 | 2.4 | + | 1250 | + |
| S6 | MNNG 4 µg/ml | (+) | 400 | 100 | 50 | (+) | 200 | - |
| | | | X Dilution | X Dilution | X Dilution | | Dilution | |
| E7 | Laar Beek SW | + | 320 | 10240 | ND | + | 40 | + |
| E8 | Textile Effluent Water | + | 80 | 1280 | 2560 | + | 80 | + |
| E9 | Congo SW - Not spiked | - | - | - | - | - | - | - |
| E10 | Congo SW - Spiked NQO | + | 256 | 2048 | ND | - | - | - |
| E11 | Congo SW - Spiked 2-AA | (+) | 64 | 256 | 512 | - | - | + |
| | | | Conc. / ng/ml | Conc. / ng/ml | Conc. / ng/ml | + / - | Conc. / ng/ml | + / - |
| Control | MMS 100 µg/ml | + | 5000 | 20 | 10 | + | 625 | + |

() = Slightly

ND = Not Determined

TABLE 1

Typical Curve response

The brightness (normalised fluorescence) of any given microtitre plate well was calculated as the fluorescence intensity (515 nm) divided by the absorbance (600 nm). The typical variation in the brightness value (BrV) with toxin dilution, in this case for MMS (100 µg/ml), is shown in figure 1. At high concentrations of the toxin, cytotoxic effects interfere with the DNA repair / GFP expression pathways themselves, and a lower than expected response is observed. Following a "peak induction", the dose response curve is clearly visible down to a "constitutive" level of GFP expression and measured concentration, which is equal in magnitude to that obtained using a "blank" solution as the sample. GFP induction is clear in both the "transformed" (T) and the "transformed + P450" (P450) strains, with relatively little induced autofluorescence noted in the "untransformed" (U) strain. The steepness of the dose response curve varies with the toxicity of the chemical analysed. A positive genotoxic response is taken as fall in induced brightness with at least two consecutive dilutions.

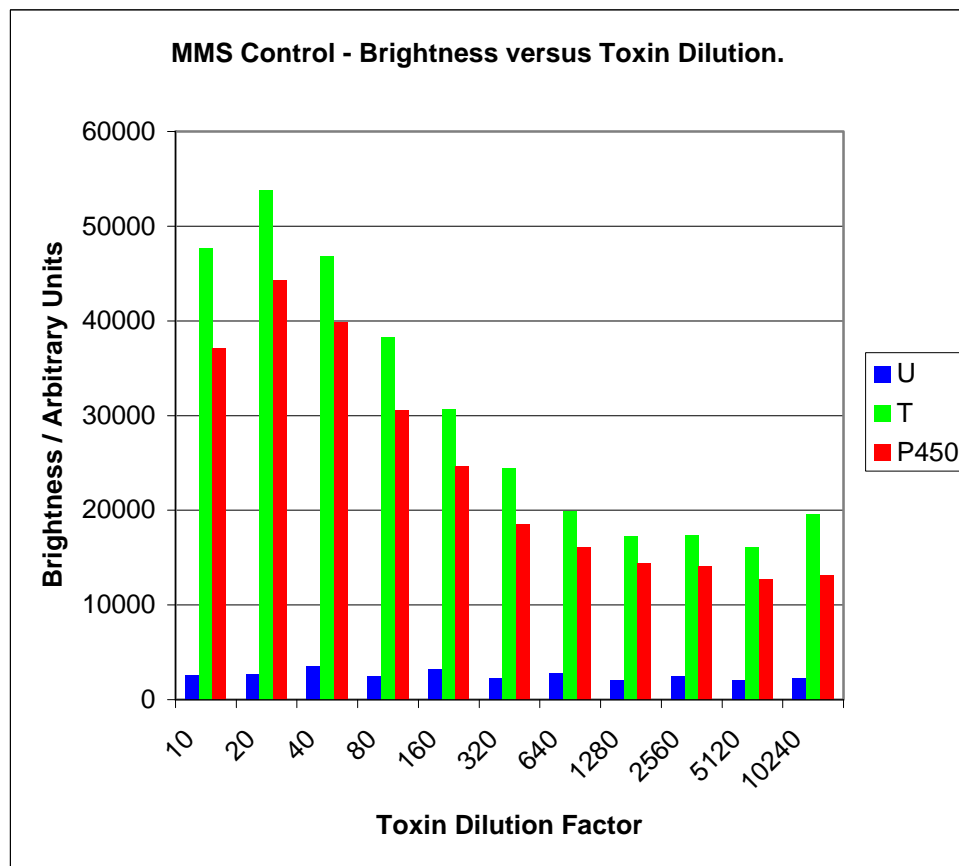


FIGURE 1

The "transformed + P450" strain shows a clear response to metabolically activated promutagens without the need for an additional metabolic activator such as S9 fraction. A typical dose response for this strain to 2-amino anthracene from an experiment carried out, in a tube assay format, just prior to the Technotox meeting is shown in figure 2.

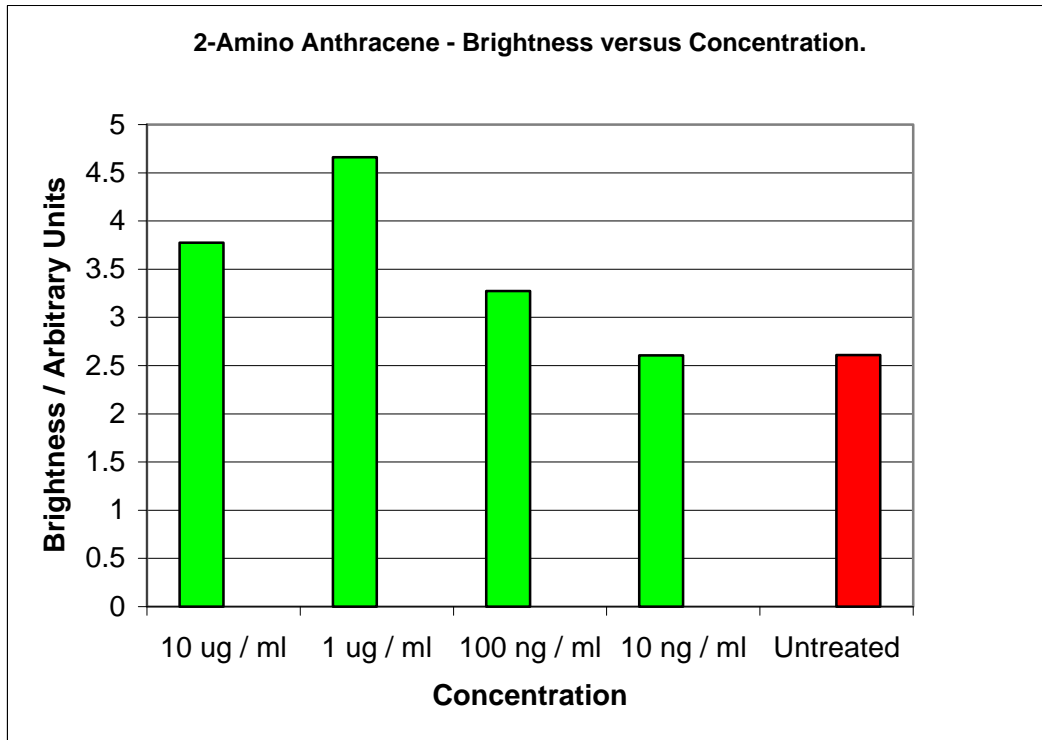


FIGURE 2.

Nature of the measurement

There are two basic quantitative genotoxicity measurements that can be obtained.

1. The induced brightness by : Peak BrV - Constitutive BrV.
2. The induction ratio by : Peak BrV / Constitutive BrV.

The following summary of the induction results shown in figure 3 gives the induced brightness above the baseline of the constitutive brightness. Subsequent fluorescence results presented graphically in this report show the absolute brightness measured, such that the brightness of the various strains can be compared and the dose response range down to the constitutive level of expression can be readily seen. Comparing induced brightness, rather than an induction ratio, gave a clearer indication of the presence and magnitude of a genotoxic response, as demonstrated in the following discussion.

Automatic scaling has been used in the presentation of data as performed by the BMG plate reader. This affects the maximum scaling on graphs but allows easy comparison of result profiles. Differences in starter culture cell densities caused some quantitative but not qualitative effects, which are noted where relevant.

Induced brightness and induction ratio

Table 2 shows the induced brightness and induction ratio observed for each of the supplied samples. The data is shown graphically in figures 3 and 4.

| Sample | ID | Induced BrV | | | Induction Ratio | |
|--------|------------------------|-------------|-------|-------|-----------------|------|
| | | U | T | P450 | T | P450 |
| S1 | 4-NQO 5 µg/ml | 849 | 13879 | 991 | 2.03 | 1.09 |
| S2 | 4-NQO 0.1µg/ml | 207 | 1397 | 1685 | 1.05 | 1.08 |
| S3 | MNNG 400 µg/ml | 8130 | 18616 | 7385 | 2.40 | 1.65 |
| S6 | MNNG 4 µg/ml | 365 | 8009 | 8916 | 1.60 | 1.83 |
| S4 | 2-AA 4 µg/ml | 198 | 1341 | 1584 | 1.08 | 1.15 |
| S5 | 2-AA 200 µg/ml | 1223 | 8344 | 4524 | 1.43 | 1.32 |
| E7 | Laar Beek SW | 140 | 19477 | 24601 | 2.52 | 3.09 |
| E8 | Textile Effluent Water | 701 | 6235 | 3933 | 1.50 | 1.34 |
| E9 | Congo SW - Not spiked | 480 | 557 | 408 | 1.04 | 1.03 |
| E10 | Congo SW - Spiked NQO | 273 | 600 | 2267 | 1.04 | 1.19 |
| E11 | Congo SW - Spiked 2-AA | 40 | 2030 | 7473 | 1.15 | 1.60 |
| MMS | MMS Control | 1496 | 37682 | 31572 | 3.33 | 3.49 |

TABLE 2

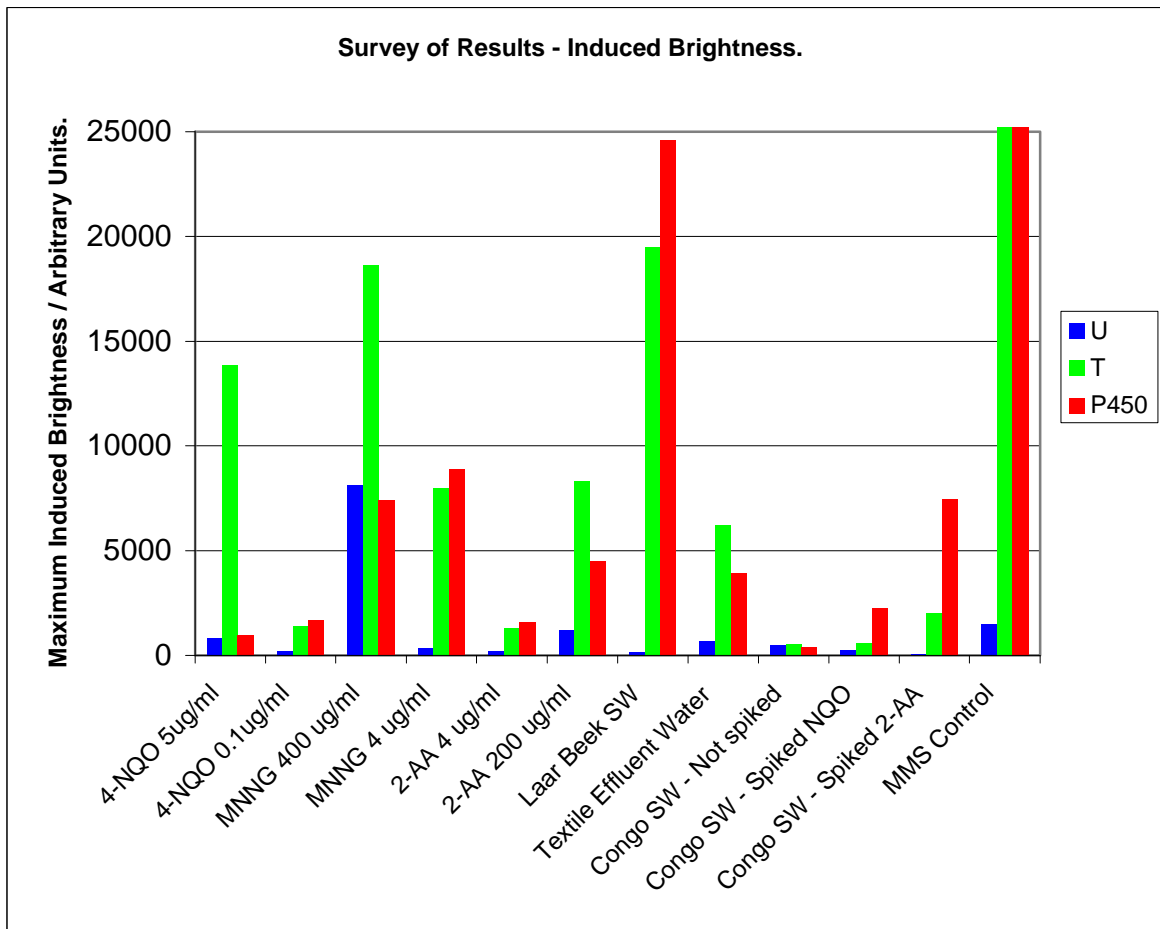


FIGURE 3

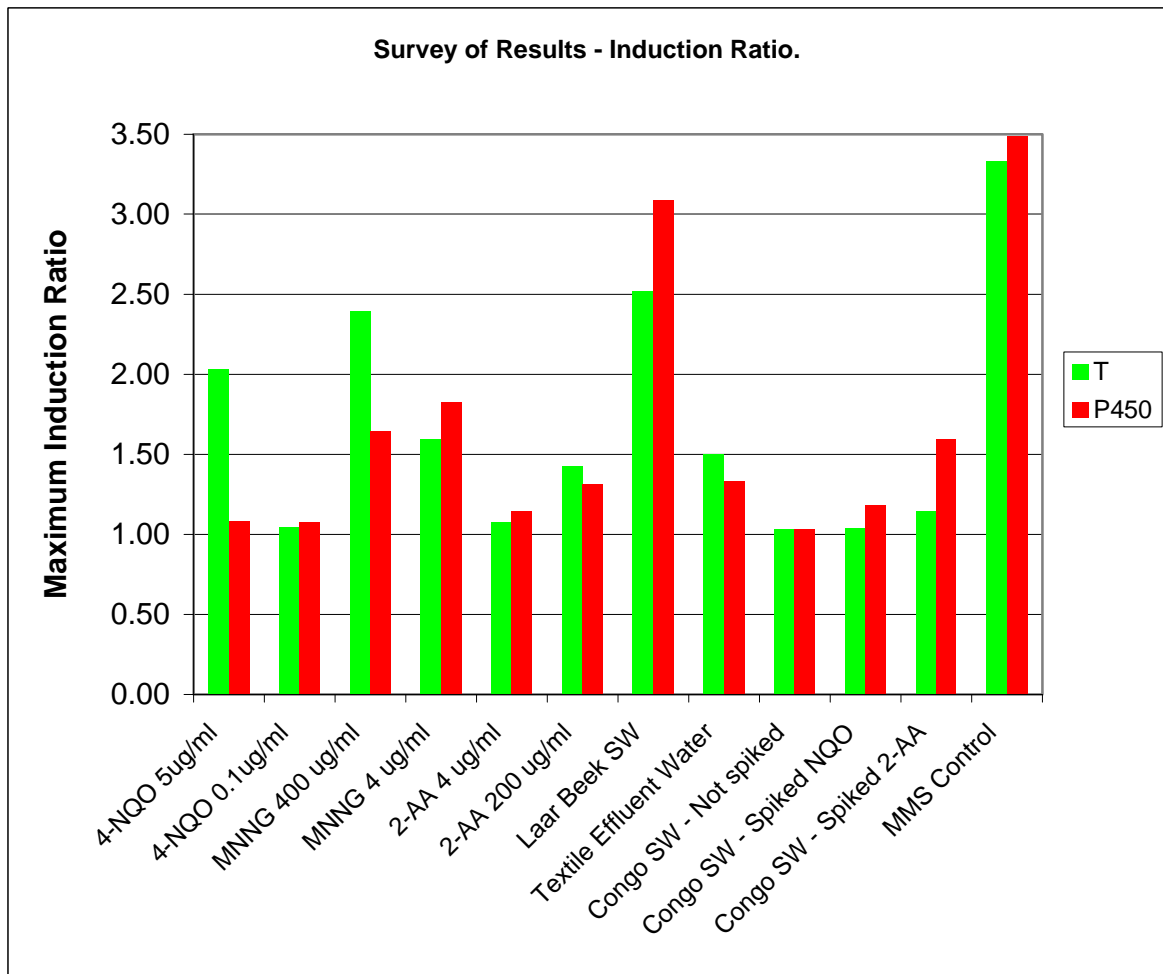


FIGURE 4

Observation of Cytotoxic Effects

Since each sample well in the microtitre plate contains the same initial concentration of yeast cells, the rate of increase in cell density, as well as the final cell density achieved are indications of the cytotoxicity of the sample examined. For convenience, in the experiments at VITO, the final cell density achieved after the 17 hours of the experiment was used to observe cytotoxic effects. The results from the analysis of 400 $\mu\text{g/ml}$ MNNG (sample S3) for example, shown in figure 5, clearly demonstrate the effect of sample cytotoxicity. The wells were inoculated with a starting cell density of 0.2. In this example, a dose response curve is seen up to 640 x dilution of the sample, after which the final cell density achieved becomes constant.

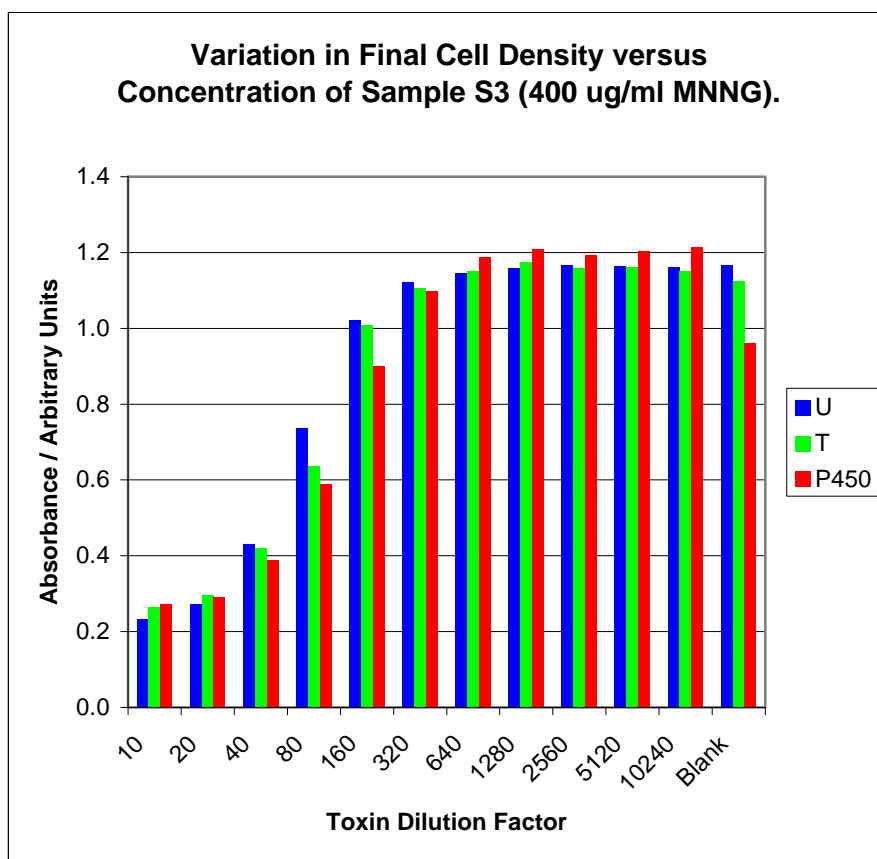


FIGURE 5

To compare cytotoxicity between the samples provided, table 3 and figure 6 show the final cell densities achieved in each microtitre well containing 10, 20 and 40 x dilutions of the test sample. The starting cell density in each case was 0.2, and the mean final cell density for dilute samples, and blanks where no cytotoxicity was observed, was approximately 1.2. The densities quotes are an average across all the strains (U, T and P450) at a given concentration.

| Sample | ID | Ave. Final Cell Density / Toxin Concentration | | | Cytotoxic |
|--------|------------------------|---|---------------|---------------|-----------|
| | | 10 x Dilution | 20 x Dilution | 40 x Dilution | |
| S1 | 4-NQO 5 µg/ml | 1.02 | 1.13 | 1.19 | + |
| S2 | 4-NQO 0.1 µg/ml | 1.24 | 1.21 | 1.22 | - |
| S3 | MNNG 400 µg/ml | 0.26 | 0.29 | 0.41 | + |
| S6 | MNNG 4 µg/ml | 1.11 | 1.27 | 1.22 | (+) |
| S4 | 2-AA 4 µg/ml | 0.96 | 1.05 | 1.02 | + |
| S5 | 2-AA 200 µg/ml | 0.73 | 0.91 | 1.10 | + |
| E7 | Laar Beek SW | 0.99 | 1.08 | 1.32 | + |
| E8 | Textile Effluent Water | 0.77 | 0.85 | 0.98 | + |
| E9 | Congo SW - Not spiked | 1.21 | 1.18 | 1.15 | - |
| E10 | Congo SW - Spiked NQO | 1.23 | 1.18 | 1.16 | - |
| E11 | Congo SW - Spiked 2-AA | 1.20 | 1.16 | 1.15 | - |
| MMS | MMS Control | 1.04 | 1.07 | 1.12 | + |

TABLE 3

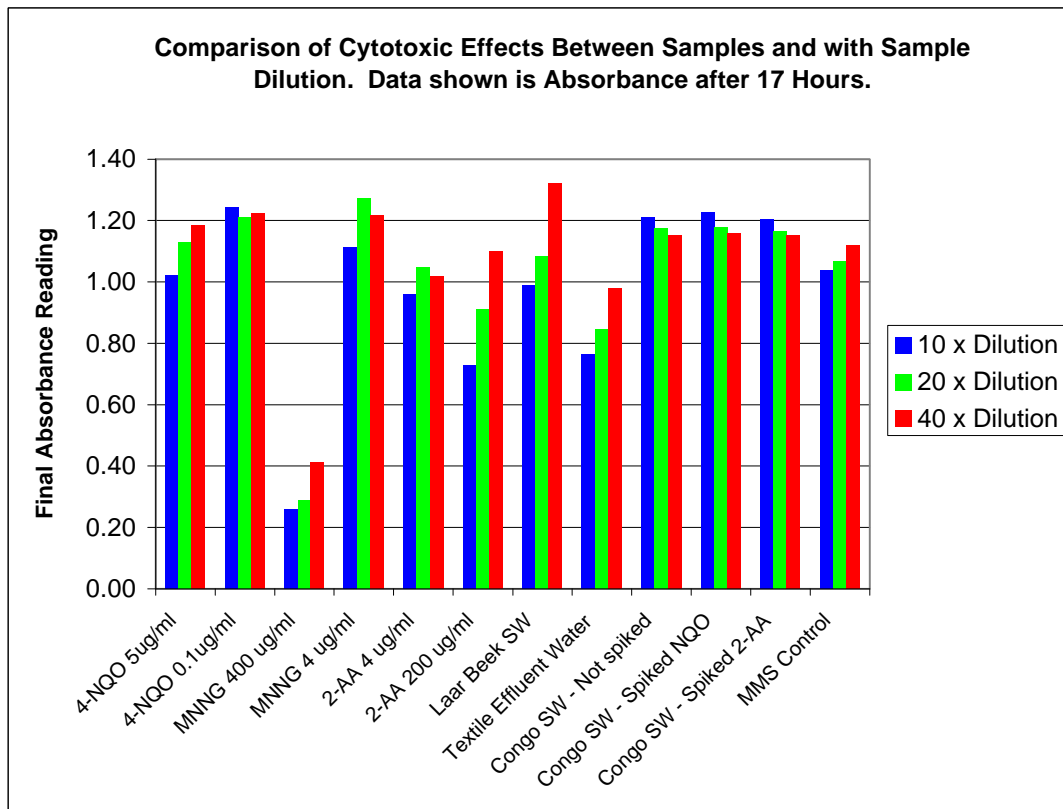


FIGURE 6

Further observations.

1. Degradation of the environmental samples.

Clear genotoxicity and cytotoxicity was evident in the polluted environmental samples E7 and E8 (surface water from Laar Beek, and Textile Industry Effluent respectively) on the first day of testing. Despite keeping the samples on ice whilst sampling and re-freezing to -20°C overnight, when they were re-tested on the second day the observed genotoxicity and especially the cytotoxicity had virtually disappeared. This suggested bacterial or chemical degradation of the toxic elements of the sample upon thawing and exposure to oxygen. The change is shown in the following figures 7 and 8, for Laar Beek surface water (sample E7).

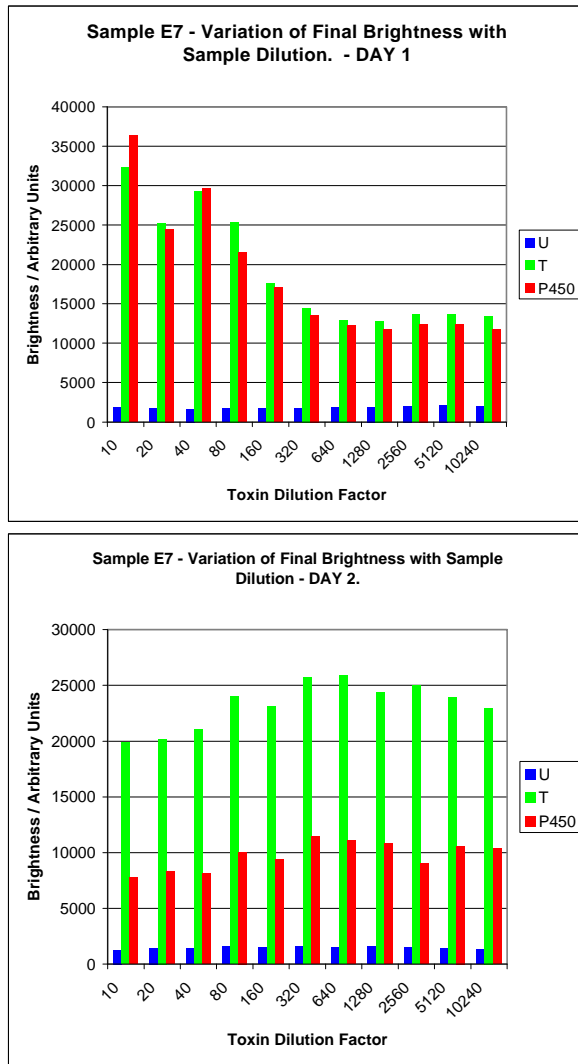


FIGURE 7. Comparison of observed genotoxic response on day 1 and day 2.

(Experimental Note: The response of the particular P450 strain culture used on day 2 was low compared to the previous day, and the T-strain response for all samples. However, both strains showed clear genotoxic induction with other samples and the control on both days).

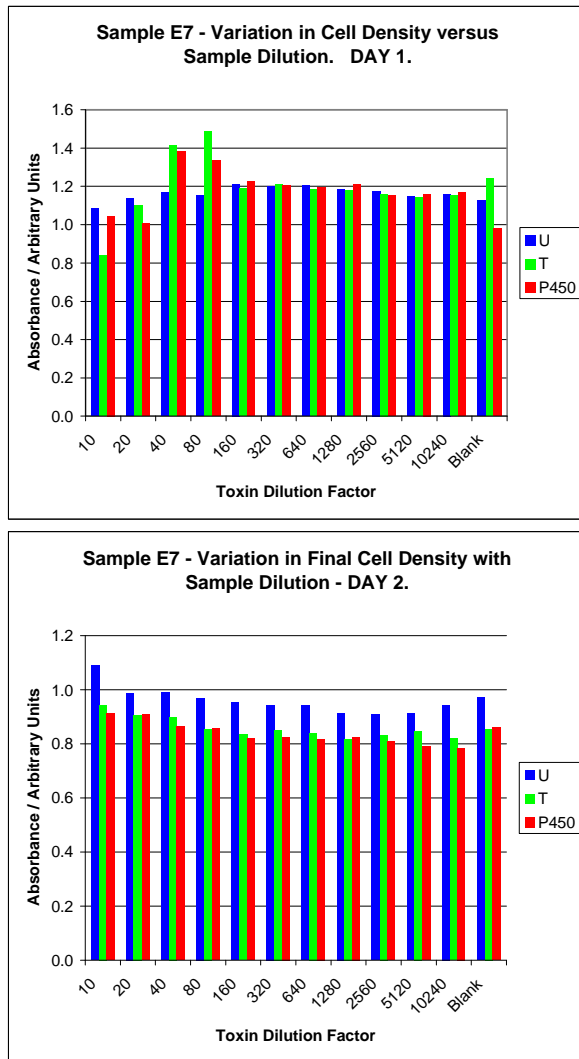


FIGURE 8. Comparison of observed cytotoxic measurement on day 1 and day 2.

2. Faster response from the transformed "P450" strain.

On the first day of testing, readings were taken at 4 and 17 hour time points. In most cases, after 4 hours, clear dose response curves could be seen in the transformed "P450" strain, whilst a response was absent in the "transformed" strain. After 17 hours, the genotoxic responses, where evident, were then clearly visible in the "transformed" strain. Interestingly in some cases, such as with MMS (see figure 9) the original response from the "P450" strain was still evident at 17 hours, whilst in other cases such as for 5 $\mu\text{g}/\text{ml}$ NQO (sample S1), as shown in figure 10, the "P450" response was obscured, perhaps by the constitutive expression of the newly grown cells, although this warrants further investigation.

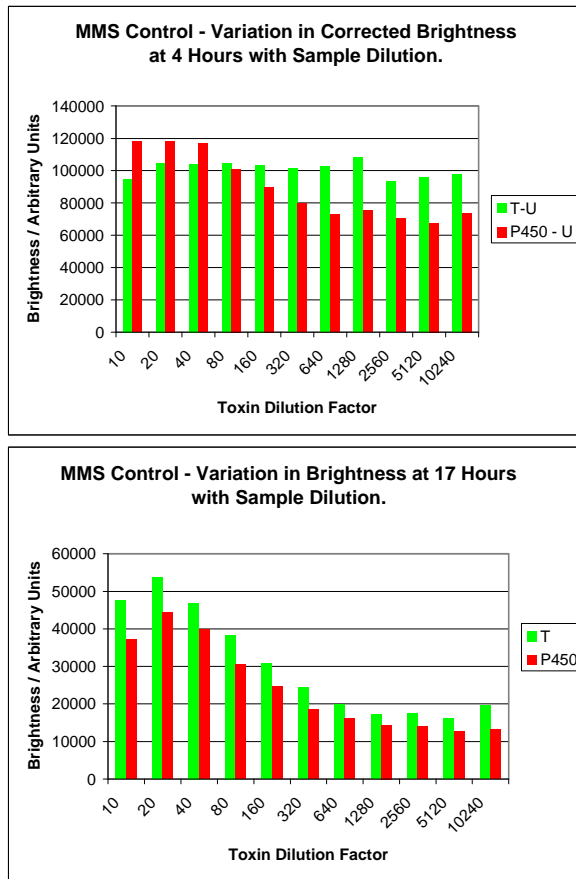


FIGURE 9

(Experimental Note: The plate reading instrument calibrates intensity on the brightest well on the plate. Therefore the absolute readings at 4 and 17 hours cannot be directly compared, rather the trends of brightness with sample dilution at each time point.)

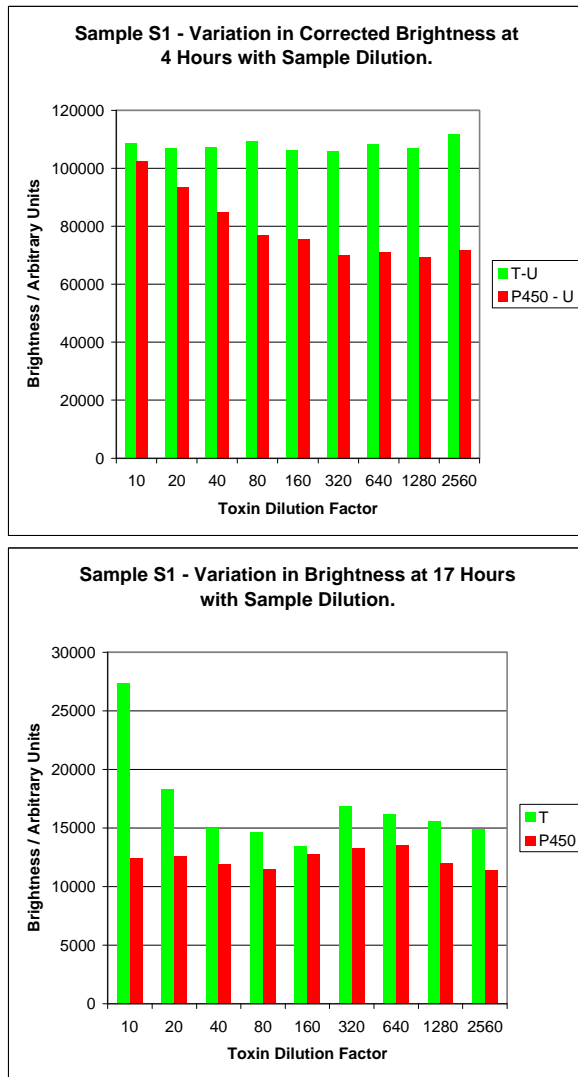


FIGURE 10

3. P450 Effect.

No S9 fraction was used in the experiments, however a small genotoxic response could be observed for 2-amino anthracene both with the "transformed" and "transformed + P450" strain. In general the dose response was clearer in the "transformed + P450" strain, although this warrants further rigorous investigation.

Genotoxic analysis using the portable instrument

In addition to the microtitre plate assay format, a prototype portable monitoring instrument was brought to VITO and tested for the first time. (See figure 11).

The instrument uses an ultra-bright blue light emitting diode (LED) as its light source, which illuminates the sample chamber containing either an acrylic cuvette or a glass semi-micro flow cell depending on the sample size available. At right angles to the light path from the LED, on either side of the flow cell, two sensitive light detectors are mounted. By the use of appropriate filters one detector measures green GFP fluorescence, whilst the second measures scattered blue light as a nephelometric measurement of cell density. The instrument runs from 4 internal PP3 batteries,

which provide power for approximately 6 hours continuous use or > 360 individual sample readings. Signals from each light detector are output to a lap-top PC where the brightness (normalised fluorescence) of the cell culture is calculated by dividing total fluorescence by cell density.

In the experiments at VITO, the contents of a microtitre well from the middle of the dilution range for each sample after 17 hours incubation at room temperature were tested. The well contained 150 μ l of liquid with yeast cells which had grown to a density of approximately 1.0 in the presence of the sample. In a clean cuvette, the contents of the well was diluted by the addition of 1 ml of distilled water, and injected into the flow cell of the portable instrument by means of a syringe. The estimation of GFP fluorescence per cell was made by automatically averaging readings obtained within a 30 s period, after which the flow cell was flushed with distilled water. The brightness of each sample culture was compared to that of a control culture. Hence genotoxicity was estimated using an induction ratio. The results are given in far right column of table 1, where indication of slight genotoxicity, "(+)", represents a measured induction in the range 1 - 1.25, and a clear positive genotoxic response, "+", represents a measured induction > 1.25.

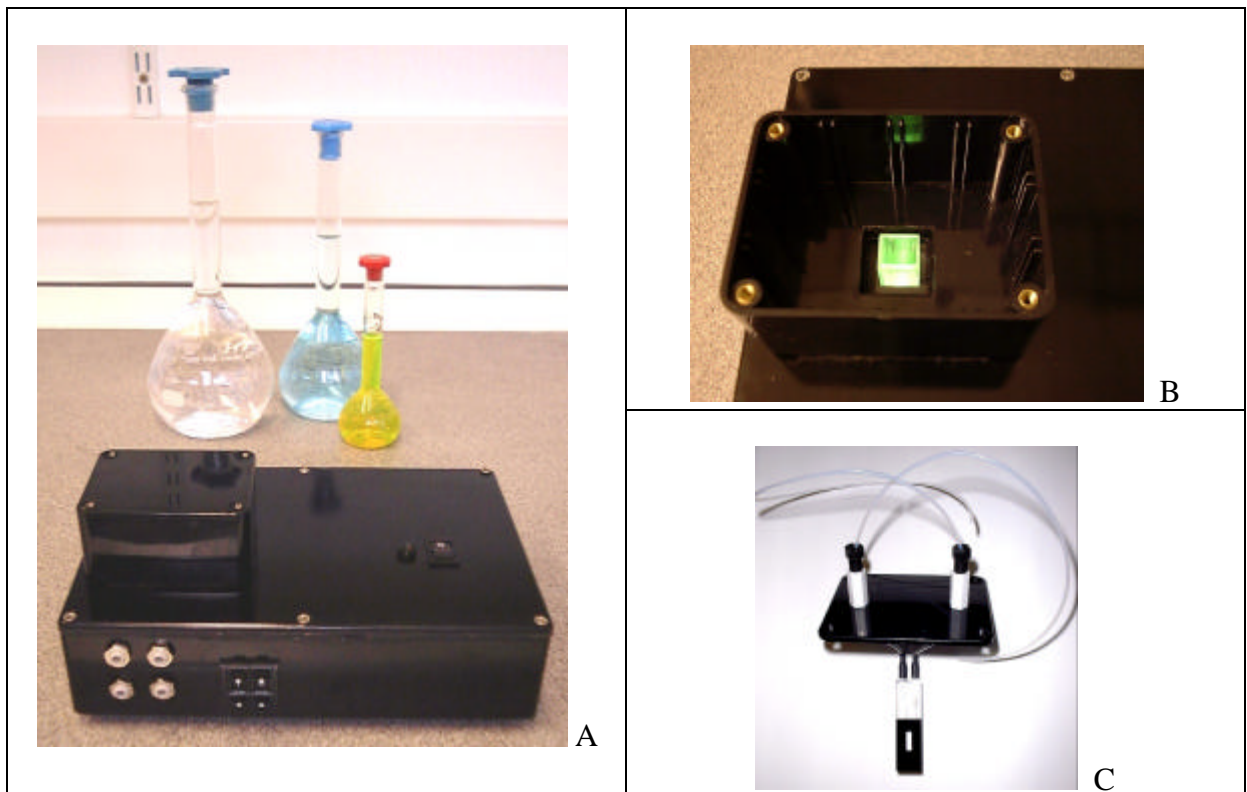


FIGURE 11 - A: Portable instrument for measuring GFP expression per cell. B: Option 1 - Cuvette measurement (1 - 3 ml of culture / sample). C: Option 2 - Flow cell measurement (< 1 ml of culture / sample).

Fluorescence microscope imaging

For illustration, figure 12 shows a fluorescence microscope image of the GM yeast cells expressing GFP, induced by exposure to MMS. Cells were viewed using a standard FITC filter set.

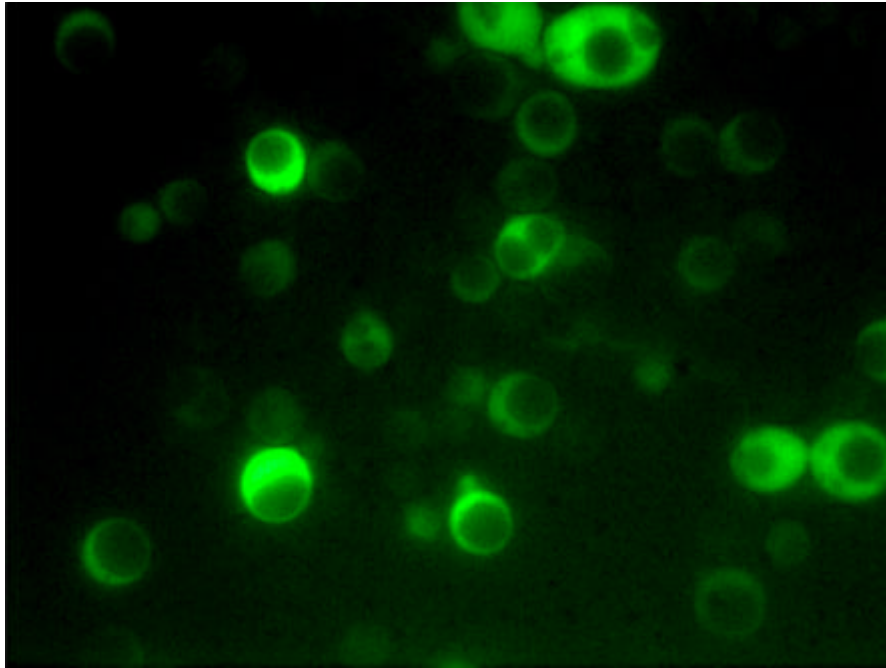


FIGURE 12.

Final perspectives

The Technotox meeting offered the first opportunity to investigate environmental samples with the EMFID RAD54-GFP test. It was also the first time that representatives of 3 of the 4 labs in this EC sponsored research programme had worked side by side in a laboratory. The Technotox Workshop was also the first time the particular micro-plate format assay had been performed, and hence this was also very much a research exercise for us.

The yeast test had previously only been used to examine pure chemicals, during the laboratory based research and development phase. The test and organisms lived up to expectation when presented with the environmental samples. Genotoxic responses were detected in the polluted and spiked surface water samples, alongside a reassuring negative response to the "clean" surface water sample. No significantly limiting interferences were evident from the use of the coloured, unfiltered and un-sterile environmental sample matrices. It was encouraging to see strong genotoxic responses from the range of chemicals supplied, especially as these included chemicals outside the range of those usually employed in our own research, as well as the use of a DMSO solvent, which we have also not routinely used before. A microtitre plate protocol was designed specifically for Technotox and was used for the first time at the meeting. The protocol proved workable and successful, and with minor changes to the sample layout on the plate, will be adopted in future work. Significantly for the team, this was a first time out for a newly developed portable device: the device and its software proved able to give a reliable yes/no output. All in all it was a most valuable exercise.

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