

A yeast-based cytotoxicity and genotoxicity assay for environmental monitoring using novel portable instrumentation

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An assay capable of simultaneously measuring both general toxicity and more subtle genotoxicity, in aqueous environmental samples, is described. The assay uses eukaryotic (yeast) cells, genetically modified to express a green fluorescent protein (GFP) whenever DNA damage, as a result of exposure to genotoxic agents, is repaired. A measure of the reduction in cell proliferation is used to characterise general toxicity producing familiar EC₅₀ and LOEC data. The assay protocol has been developed for proposed use in the field and hence employs dedicated, portable instrumentation, the development of which is described. A range of environmentally relevant substances has been evaluated using the assay, including solutions of metal ions, solvents and pesticides. Preliminary data comparing the yeast assay's response to that of a standard *Daphnia* test in the analysis of the toxicity of 34 varied industrial waste effluents are also presented. The sensitivity to a wide range of substances and effluents suggests the assay should be useful for environmental toxicity monitoring.

Introduction

There is currently a need for simple methods that can be used for the screening and monitoring of a wide range of toxic contaminants in the aquatic environment. For example, in assessing the effectiveness of waste treatment, to control the toxicity of industrial effluent discharges into surface waters, in the protection of water treatment plants from toxic influents and in hazard assessment in areas where the deliberate release of chemical armaments is suspected. Such methods are ideally required to be cost effective, rapid, portable and able to be performed by relatively unskilled personnel.

The need for the monitoring of toxicity in industrial wastes is also evidenced by the increasing legislative control over the disposal of effluents and hazardous chemicals into the aquatic environment. One of the aims of the new Water Framework Directive (2000/60/EC) which comes into effect from December 2003 is to progressively reduce the emission of hazardous substances into all ground and surface waters within the European Community (EC) and ensure stable, long term planning of protective measures. One of the ways in which the Directive will achieve this will be to include measures required under the current Integrated Pollution Prevention and Control (IPPC) Directive (96/61/EC), an integral part of which is the provision of permits to operators to discharge effluent. In order to obtain a permit, an operator must comply with a number of criteria, including the use of BAT 'Best Available Techniques' to prevent or minimise pollution, which are both technically and economically viable.¹ Significantly, to quote the IPPC technical guidance notes, "the Regulators encourage the development and introduction of new and innovative techniques that meet the BAT criteria".

In this paper we propose a novel environmental monitoring tool capable of assessing general toxicity and, more specifically, genotoxicity. The value of genotoxicity, *i.e.* a measure of subtle and heritable damage to DNA, in addition to general toxicity, as an indicator of ecological health is given credence by Annex VIII of the Water Framework Directive,² which identifies

"substances and preparations, or the breakdown products of such, which have been proved to possess carcinogenic or mutagenic properties" as one of the categories of main pollutants of concern. Concern over the threat from genotoxins was also expressed in the European Union Environment Agency report on the "Environment in the European Union at the turn of the century".³ One section of this report highlights the limited amount of toxicity and ecotoxicity data relating to chemicals used in large volumes by industry. Of these chemicals, only 60% have any genotoxicity/mutagenicity data. This lack of a provision of data for the formulation of risk assessments has been identified as a major shortfall, with the problem likely to be exacerbated by an estimated 30–50% rise in the output of large volume chemicals.

Historically toxicity testing in the context of environmental monitoring has been performed by characterising the levels of specific species, such as a heavy metal ion or pesticide, and comparing to known toxicity thresholds. A wide range of analytical techniques and sensors have been developed to achieve this. However this approach has several disadvantages. It misses unexpected toxicants that the analyst is not specifically looking for; it misses synergistic effects, whereby the risk from two or more toxic species present together is not equal to the sum of their respective toxicities; and many techniques using this approach do not take into account the bio-availability of the toxicant in its natural matrix. Tests are required to make a rapid assessment and warn of toxicity in whole environmental samples, following which more detailed and expensive laboratory analysis can be made, only when required.

In order to assess risk to biological species, the use of a biosensor is the most pharmacologically relevant approach. Many biosensors for toxicity employ a component of cells such as an enzyme, antibody or organelle.^{4–8} Although such sensors are usually rapid and extremely sensitive, they often lack robustness, generally have short shelf lives, and reproduce only a small part of the mechanism of the interaction of toxicants with biological organisms. A more relevant picture is obtained

when a whole organism is present. In environmental toxicity assessment this has been done by the use of ecologically relevant species such as *Daphnia magna*, brine shrimp and larger organisms such as fish.⁹ Such tests are expensive to perform due to the animal husbandry required to maintain populations of the test organisms, and the use of behavioural endpoints with larger organisms can be more subjective than quantitative.

Micro-organisms provide a neat solution, since they can be cultured easily and inexpensively from frozen or freeze dried stocks, and their small size, simple morphology and large surface area in relation to their size can give them increased sensitivity compared to larger, more complex organisms. Micro-organisms are also more tolerant than cellular components to sub-optimum conditions of temperature and pH. Many uses of micro-organisms in biosensors have been reported using a variety of parameters to assess the toxic effect, principally the inhibition of cell proliferation, substrate consumption or conversion, respiration and bioluminescence.^{4,10} However, optical methods of detection are by far the most convenient, non-invasive and generally the most free from interference.

Methods of toxicity testing using naturally bioluminescent micro-organisms, have centred on the marine bacterium *Vibrio fischeri*, and are well characterised and commercially available as the Microtox[™] and ToxAlert[™] systems, amongst others.^{11–13} Bioluminescence depends on respiratory metabolism and is thus linked to the metabolic status of the cell. Acute toxicity results are produced in a matter of minutes, however a significant disadvantage is that samples must be adjusted to 2% sodium chloride for osmotic protection of the marine organism.

The advent of genetic engineering has allowed the creation of genetically modified (GM) organisms whose gene function has been altered. For example the Mutatox[™] system uses a GM dark variant of *Vibrio fischeri* to detect genotoxins.^{11,14,15} Another example is Vitotox[™], commercialised by Thermo Labsystems.^{16–18} The assay system exploits the SOS-response mechanism in GM strains of the *Salmonella* bacteria, which luminesce in the presence of a DNA damaging species. The method however requires the use of large, complex microplate readers and associated technology, which are not suitable for work in the field, the assay being principally designed for high-throughput, laboratory based, screening in the pharmaceutical industry.

Unlike luminescence approaches, techniques based on fluorescence require little energy from the cell, as the presence of a fluorescent protein for example, is detected simply by illumination with an appropriate light source. One such example, for detecting cytotoxicity using a strain of *Salmonella typhimurium* genetically modified to express green fluorescent protein (GFP), has been developed by Baumstark-Khan *et al.*¹⁹ Quantification of green fluorescence gives a measurement of metabolic activity, and hence fluorescence is reduced when cells are exposed to a cytotoxic sample.

Each of these techniques are, however, based on bacteria, a prokaryotic organism. In this paper a yeast based genotoxicity and cytotoxicity test for use in environmental monitoring is presented. Unlike bacteria, yeast is a eukaryotic organism. Since humans are also eukaryotes, and share many of the same structural and biochemical characteristics in our cells, the results of the test are more relevant for human risk assessment. The yeast cells are genetically modified to express a yeast enhanced GFP under the control of a copy of the promoter from the native yeast gene *RAD54*. *RAD54* is known to be specifically upregulated by the cells in response to DNA damage, and thus on exposure to a genotoxic agent the cells become increasingly fluorescent as GFP accumulates.^{20–22} The GFP gene, originally cloned from the jellyfish *Aequorea victoria*, has the advantages of possessing favourable properties such as low toxicity, high chemical and photostability, and the

ability to spontaneously form a fluorophore upon expression without the need for additional reagents or co-factors.²³

In the assay the sample is combined with a culture of the yeast in a specialised growth medium and then incubated overnight. A single measurement is subsequently made of fluorescence and cell density. Fluorescence is used to indicate the presence, concentration or potency of a genotoxin, whilst the cell density provides a measure of cytotoxicity which restricts cell proliferation during incubation. Note that cytotoxicity is characterised by a restriction in relative total growth compared to a non-toxic control and is not a direct measure of cell death, as such it reflects the toxicity of a substance on the whole cell cycle and proliferation of the yeast. Thus the assay characterises both gross toxicity and more subtle genotoxicity. The assay has been developed in a variety of formats including a microplate based protocol for pharmaceutical screening.²⁴ This paper describes the development and evaluation of a small portable instrument and associated protocol for use in environmental monitoring. The instrument has been designed for use in an assay capable of measuring the toxicity of aqueous samples collected in the field. The assay has been assessed by analysing both a range of environmentally relevant pure compounds and a diverse selection of industrial effluents.

Materials

Yeast

A DNA repair-competent strain of the brewer's yeast *Saccharomyces cerevisiae* was employed as the host strain for a reporter of DNA repair activity (the "test" strain). The reporter consisted of a fusion of the DNA damage-inducible promoter from an endogenous DNA repair gene, *RAD54*, with a gene encoding a yeast enhanced green fluorescent protein (yEGFP). Details of this strain and reporter have been published elsewhere.^{20,21} yEGFP has an excitation maximum at 490 nm and emission maximum at 517 nm. A second "control" strain was used to correct for any effects of the presence of the plasmid on the extent of proliferation and cellular fluorescence. The control strain contained a disabled reporter plasmid, and thus was unable to express GFP despite being identical to the test strain in every other way.

Chemicals

The inorganic metal salts used were obtained from Fluka (Gillingham, UK) and were all of analytical grade, >98% pure. For increased solubility, hydrated sulfates of cadmium, copper, nickel and zinc, and chlorides of lead, mercury and chromium were used. All concentrations quoted are with respect to the metal ion. Organic compounds were obtained from Sigma Aldrich (Poole, UK) and of the highest purity available. Due to its inherent instability, sodium hypochlorite was purchased from the manufacturer (VWR Ltd. Poole, UK), stored in the dark and used without delay. The stock standard was rated at 10% available chlorine and concentrations quoted here are as ppm available chlorine.

Assay protocol

Advance preparation

Prior to the test, cultures of the test and control yeast strains were grown from inocula stored in a freezer, over 2 days at 30 °C in shaken 250 ml sterile flasks. The culture density was ascertained and the culture transferred to sealed falcon tubes for storage and transportation. These stock cultures were stored at approximately 4 °C and could be used for up to a week. In this way, by producing cultures on a weekly basis, yeast stocks were always available for testing without

unnecessary delay. The optical density (OD) of the culture was typically in the range 4.5–6.5 (600 nm, 1 cm path length). Prior to a test, an aliquot of the stock yeast culture was added to fresh growth medium to form the “yeast reagent” for the test. The volume of the aliquot of yeast culture was selected such that the yeast cells were present at a concentration of 0.2 OD in the yeast reagent. The growth medium used was a well defined minimal medium (nominally pH 5.5–6.0), which exhibits low autofluorescence,²⁵ from which the excess concentrations of buffer salts, principally phosphates were omitted. This modification was made to reduce the precipitation of metal ions from sample matrices, which limited their bio-availability, and to allow the sample’s pH characteristics to contribute to the overall toxicity evaluation of the sample. All components of the medium used in the “yeast reagent” were of twice the required concentration, such that upon 50 : 50 v/v dilution with the test sample, the test solution would contain the sample diluted twofold, 0.1 OD yeast cells and the optimum concentration of growth medium.

Determination of cytotoxicity and EC₅₀

For cytotoxicity assessment, 1 ml of each sample dilution to be tested was combined with 1 ml of the yeast reagent in a pre-labelled, disposable cuvette. No further reagents were required for the assay. Either the test or control strains can be used for cytotoxicity assessment since cellular fluorescence is not recorded. For this work the control strain was used. At least one non-toxic control was also prepared in which pure water was used as the test sample. The cuvettes were acrylic, 3 ml in volume and had 4 optical windows (Sarstedt Ltd, Leicester, UK). The cuvettes were sealed with an adhesive breathable membrane (“Breathasy”, Diversified Biotech, Boston, MA, USA) cut to an appropriate size, and left overnight to incubate, ideally at 25–30 °C.

Before measurement the contents of the cuvette were thoroughly mixed by shaking or repeated pipette aspiration to re-suspend the yeast cells. The cuvette was placed in the reader, described later, and the yeast cell density was determined by a nephelometric (light scatter) measurement. The assay therefore determines the ability of the sample, by virtue of its toxicity, to restrict yeast cell proliferation.

For environmental samples, such as industrial effluents, which contained particulate matter, comparative cuvettes were prepared containing the corresponding dilution of the sample and growth media alone, without cells. Optical density values determined from these cuvettes can be subtracted from the respective assay cuvettes to correct for sample turbidity. Thus:

Cell culture density measurement (control strain) = $D_C - D_X$

Cell culture density measurement (test strain) = $D_T - D_X$

Where D_C and D_T are the nephelometric signals measured from the sample with the control and test yeast strain respectively, and D_X is the nephelometric signal from the control containing the sample and growth media only. This correction worked well since insoluble particulate matter commonly found in environmental samples is largely unaffected by the presence of the yeast cell culture.

A basic qualitative (highly toxic, toxic or non-toxic) result can be obtained from a single cuvette measurement by comparison to a blank, non-toxic control. However to obtain a quantitative result an EC₅₀ was determined. To determine an EC₅₀ value the test sample was diluted to form a linear range of typically 8 to 10 concentrations and each was tested separately. A parametric curve was fitted to the data describing the variation of final cell density with sample dilution. From this curve the predicted sample dilution corresponding to 50% cell growth (or proliferation) was calculated to be the EC₅₀ (effective concentration giving 50% response). 50% cell growth is equivalent to a density reading half way between the culture density at the start of the assay

and the density achieved in the non-toxic control. Note, that by using a culture population of several million cells, the cytotoxicity evaluation should be more reproducible and easily quantified than comparative assays such as *Daphnia magna* which use 5 to 10 organisms per test.

Determination of LOEC

From the data obtained during the cytotoxicity assessment stage, a LOEC (lowest observed effect concentration) value can also be obtained, equal to the concentration at which a toxic effect produces 10% less cell proliferation than observed in the non-toxic control. This figure takes into account the natural variation in proliferation observed when making up replicate control cultures, *i.e.* three times the standard deviation in final cell density.

Genotoxicity assessment

For genotoxicity assessment, the process described above was repeated using a linear series of sample dilutions across the sub-cytotoxic range determined for the sample. Both the test and control strains were used with each sample dilution. Only sample concentrations below the EC₅₀ value were counted since at severe levels of cytotoxicity the loss of cell integrity can lead to non-specific DNA damage. Furthermore, at such concentrations, cytotoxicity is the more relevant end-point.

Cuvettes were placed in the reader and measurements of cell density and sample fluorescence were simultaneously obtained. Thus, F_T is the fluorescence measurement from the test strain culture, and F_C that from the control strain culture, at any given sample concentration.

The induced GFP fluorescence was calculated from the difference between the “brightness” of the test and control strains, in comparison to a non-toxic control. “Brightness” corresponds to the fluorescence reading normalised for cell density, since the toxicity of the sample can affect the final cell density to different extents, depending on its mode of action.

Thus the measured brightness may be expressed as:

Brightness (test strain) = $BR_T = (F_T)/(D_T - D_X)$

Brightness (control strain) = $BR_C = (F_C)/(D_C - D_X)$

Any autofluorescence from components of the test sample, or endogenous autofluorescence that is induced within the yeast cell, is corrected for by subtracting the brightness value observed for the control strain (which does not express GFP) from that of the test yeast strain. Thus the corrected brightness reading for any particular sample dilution is expressed as $BR_T - BR_C$. Since the autofluorescence of an environmental sample is significantly affected by the presence of yeast cells, which alter the pH and ionic conditions of their medium during proliferation and can metabolise components of the sample, so the autofluorescence correction is made by subtraction of control and test culture brightness values, rather than using direct fluorescence measurements of the sample alone. It is appreciated however, that satisfactory results for genotoxicity may be obtained using the test yeast strain only, without corrections as described, in cases where the test sample is not significantly autofluorescent or optically dense.

A positive genotoxicity result is obtained if at least one sample concentration in the series produces a brightness reading >30% higher than the blank (*i.e.* an induction of 1.3). This threshold was previously determined as greater than three times the standard deviation in brightness from a series non-toxic controls in the cuvette assay. A dose-dependent response provides further evidence of a positive result. A genotoxicity LOEC is the lowest concentration which produces an induction of greater than 1.3.

Standards

To ensure that the yeast strains are behaving as expected and that the results obtained are both credible and reliable, standard compounds were included in each trial. Two concentrations of 3,5-dichlorophenol (3,5-DCP) were tested (15 and 7.5 mg L⁻¹) to check for a dose dependent cytotoxic response, and two concentrations of methyl methanesulfonate (MMS), were tested (0.005 and 0.00125% v/v) to check for GFP induction and thus a dose dependent genotoxic response.

Portable instrumentation

The portable instrumentation developed for this assay combines a fluorescence spectrometer and nephelometric detector. The instrumentation was nicknamed the Yeast Environmental Toxicity Indicator or YETI for short. The instrument shown in Fig. 1, uses an ultra-bright light emitting diode, LED, (473 nm, 2000 mcd, RS Components Ltd. Corby, UK) to illuminate the yeast cell suspension housed in the acrylic cuvette. The cuvette is placed into a holding chamber within the unit, which is light-tight when the hinged lid is replaced. Light scattered by the yeast cells in suspension is detected using a silicon photodiode, SPD, (S5591, Hamamatsu Photonics Ltd., Enfield, UK) mounted at right angles to the direction of illumination. This signal is proportional to the concentration of yeast cells present. Cellular fluorescence is measured using a miniature photomultiplier tube, PMT, with an integral microelectronic amplifier (H5784, Hamamatsu Photonics Ltd) also mounted at right angles to the direction of illumination, opposite the SPD.

A set of filters was developed for this optical arrangement to pick out the wavelength region for GFP excitation from the broad LED emission spectrum, and to effectively filter out scattered blue light from the green fluorescence detected by the PMT. The excitation light from the LED was passed through a combination of a band-pass filter (475 nm, 40 nm band-width) and a blue, Schott glass, short-pass filter (BG3, 3 mm thick). Scattered light was measured after passing through an interference filter (470 nm, 10 nm half-bandwidth) and a neutral density filter (OD = 1) to reduce the intensity 10 fold. Green fluorescence was measured after the light has passed through a combination of an interference filter (515 nm, 10 nm half-bandwidth) and an orange, Schott glass, short-wave cut-off filter (OG515, 9 mm thick). All filters were obtained from Comar Instruments (Cambridge, UK) with the exception of the band-pass filter (475RDF40) which was purchased from Glen Spectra (Stanmore, UK). All filters and detectors are housed

within appropriately sized, tubular optical mounts fitted into a black plastic housing (constructed in-house), which also forms the sample chamber to house the cuvette. The spectra characteristics of the optical filter set, the overlap with the LED emission profile and the GFP fluorescence excitation and emission spectra are shown in Fig. 2.

In addition to the optical detectors further electronic circuitry was provided for signal processing and power supply and regulation. All electrical components are powered by 4 internal metal hydride rechargeable batteries, which provide sufficient power for in excess of 12 hours continuous use or approximately 800 single readings.

Signals from the optical detectors were smoothed, offset and amplified using purpose built op-amp circuits and passed to a small analogue to digital converter (ADC-11, Picotechnology Ltd. Cambridge, UK). The ADC was connected to a lap-top computer and data from the PMT and SPD collected and averaged using PicoLog software (Picotechnology). In a later prototype these readings could also be directly viewed on digital displays. Data is entered directly into an automatic data analysis template, written in-house using Microsoft Excel and Jandel SigmaPlot software. The software provides EC₅₀, LOEC and genotoxicity assessment.

Results and discussion

Instrument sensitivity

The sensitivity of the instrument for fluorescence detection was examined using solutions of fluorescein, in 0.1 M phosphate buffer at pH 7.0. At optimum sensitivity settings, a linear calibration was achieved over the concentration range 0 to 1.6 × 10⁻¹¹ M, with a limit of detection of 4 × 10⁻¹² M, equivalent to the blank plus 5 times the standard deviation of the blank, with a correlation coefficient of 0.9991 (*n* = 9). The sensitivity is thus more than adequate for the detection of yeast cell fluorescence which was made at a programmed setting equivalent to a 25 fold reduction in sensitivity compared to the fluorescein calibration.

The characteristics of the detection of scattered light in the instrumentation was examined using white latex microspheres as a mimic for the yeast cells (5 μm diameter, Polymer Laboratories, Church Stretton, UK). A linear relationship between scattered light and percent suspended solids of microspheres up to an equivalent OD of 3 (600 nm, 1 cm path length) was obtained, which adequately covers the range of cell densities achieved in the overnight incubation. Thus, SPD signal (mV) = 21.13 × concentration of suspended solids

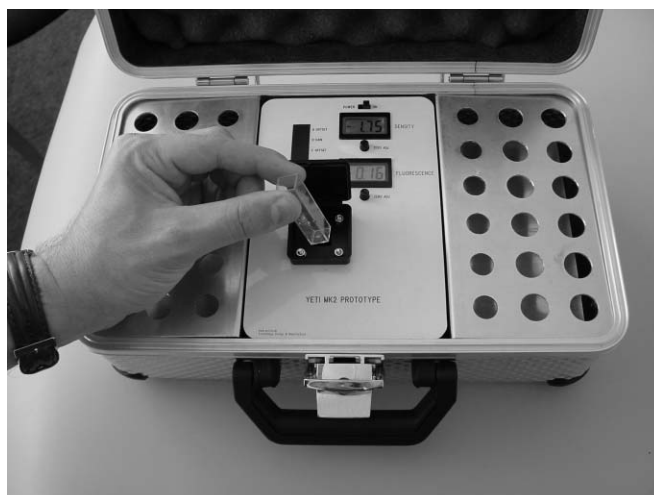


Fig. 1 Photograph of the current prototype of the YETI-cytotoxicity and genotoxicity sensing portable instrumentation. The instrument is packaged in a robust case with space for storage of sample cuvettes.

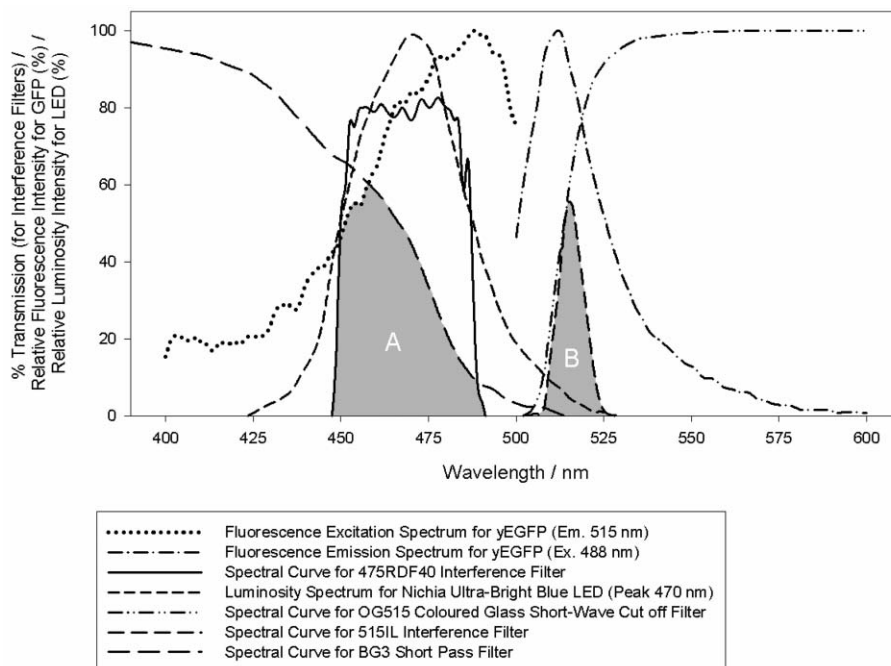


Fig. 2 Illustration of the spectral overlap of the optical filters, yEGFP excitation and emission and LED light output. Area A indicates the range and magnitude of the excitation spectrum available, and Area B the range and magnitude of the emission spectrum detected.

(% w/v), $r = 0.9988$ ($n = 10$). Measuring the optical density of the prepared suspensions using standard absorbance techniques gave a linear relationship only up to an OD of 1.

Standard results

Initially the protocols were tested with standard chemicals of known activity. For cytotoxicity characterisation the commonly used standard, 3,5-dichlorophenol (3,5-DCP), was employed. For genotoxicity, methyl methanesulfonate (MMS), a known genotoxic, DNA alkylating agent was used. Fig. 3 shows the characteristic s-shaped curve for the relationship between final cell density and concentration of the test compound. The EC_{50} and LOEC points are marked. An EC_{50} of 7.5 mg L^{-1} was obtained which is within the generally recognised range for toxicity tests of 5 to 30 mg L^{-1} .²⁶ MMS produced a clear, dose dependent, induction in fluorescence of the test strain due to the expression of GFP, exceeding the threshold value for a positive genotoxic evaluation, as shown in Fig. 4. Both 3,5-DCP and MMS consistently give reproducible results with this assay and hence have been adopted as the standard positive controls for cytotoxicity and genotoxicity respectively.

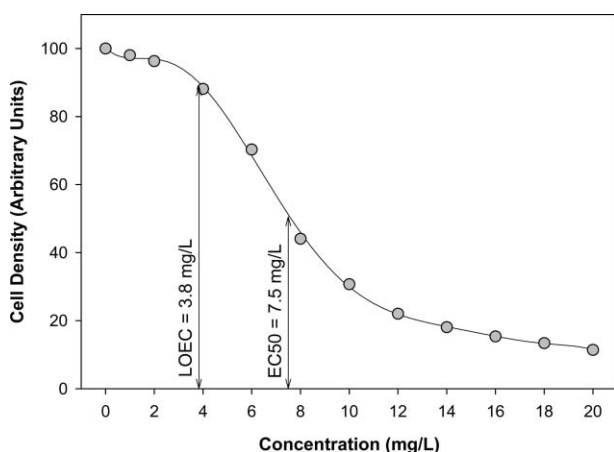


Fig. 3 Cytotoxicity profile for the standard 3,5-dichlorophenol (3,5-DCP).

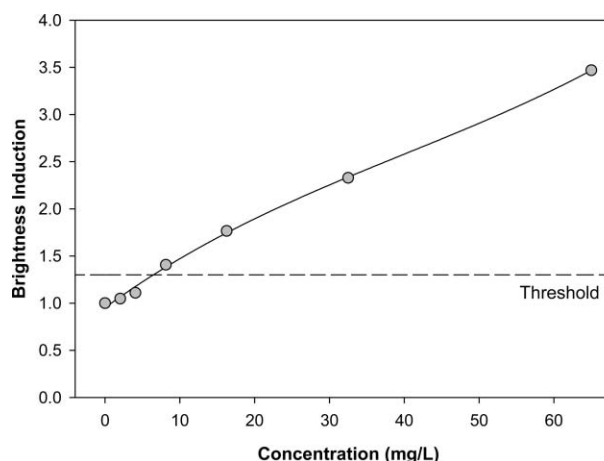


Fig. 4 Genotoxicity profile for the standard methane methylsulfonate (MMS).

Pure compounds

The assay was validated against a range of environmentally relevant compounds, including heavy metal ions, solvents, pesticides and other organic and inorganic compounds. The results obtained are summarised in Table 1.

Pure compounds – cytotoxicity

An example cytotoxicity result is shown in Fig. 5 for copper(II) ions. The assay was most sensitive for cadmium and copper ions, both of which have historically been widely used in fungicidal preparations, and yeast are unicellular fungi. For the same reason the assay was also highly sensitive for cycloheximide. It has been estimated, for example, that fungicides account for around 60% of the total agricultural use of pesticides in Atlantic Canada,²⁷ and therefore yeast should prove a sensitive organism for pesticide detection. The assay was also reasonably sensitive to the remaining compounds tested. These included sodium dodecyl sulfate as a model surfactant compound, sodium hypochlorite (common bleach) as a model household cleaning agent and nitrogen mustard as an example organic chemical warfare agent, the latter

Table 1 Summary of cytotoxicity and genotoxicity results for the pure compounds tested

Class	Compound	Cytotoxicity EC50 (mg L ⁻¹)	LOEC (mg L ⁻¹)	Genotoxicity Evaluation ^a	LOEC (mg L ⁻¹)	Solvent
Metal Ions	Cadmium(II)	0.032	0.019	N	—	Water
	Copper(II)	0.052	0.023	N	—	Water
	Chromium (VI)/Dichromate	0.55	0.041	N	—	Water
	Mercury(II)	0.47	0.18	N	—	Water
	Nickel(II)	21	1.7	P	14	Water
	Chromium (III)	28	4.7	N	—	Water
	Zinc(II)	126	21	N	—	Water
	Lead(II)	—	30	N	—	Water
Solvents	Methanol	24000	3700	N	—	Water
	Ethanol	25000	4200	P	24000	Water
	Dimethyl sulfoxide	41000	9200	P	22000	Water
Pesticides	Cycloheximide	0.015	0.0023	N	—	Water
	2,4-D/2,4-Dichlorophenoxyacetic acid	55	7.8	N	—	Water + 1% DMSO
	Paraquat/Methyl viologen	101	11	P	30	Water
Others	Nitrogen mustard/Mechlorethamine HCl	0.32	0.047	P	0.2	Water
	Sodium hypochlorite	2.6 ^b	1.5 ^b	N	—	Water
	3,5-Dichlorophenol	7.5	3.8	N	—	Water
	Sodium dodecyl sulfate (SDS)	33	3.3	N	—	Water

^a Genotoxicity evaluation, N = negative and P = positive. ^b ppm available chlorine

indicating a possibility of using the technology for military applications. The assay was considerably less sensitive for common organic solvents, although the mg L⁻¹ concentrations quoted represent an EC₅₀ of approximately 3% and LOEC of less than 1% v/v. Reducing the phosphate ion concentration of the media allowed the analysis of all metal ions without significant precipitation, with the exception of lead(II). In this case a separate series of sample dilutions were prepared containing lead with growth medium alone and the density readings obtained subtracted from those containing the yeast cells in growth medium. The correction was effective, revealing a distinct toxicity profile, however the low bioavailability of the lead ions in the media reduced its toxicity such that only a LOEC could be obtained within the soluble concentration range.

A number of other cytotoxicity assays using yeast as the test organism have been previously reported. Iwahashi *et al.* reported a multi-endpoint bioassay system to characterise environmental pollutants based on their effects on growth ability, viability, stress protein induction and various mutations.²⁸ The protocols used involved colony growth, and counting on agar plates or density estimations in micro-well plates, hence require a degree of expertise in such techniques and use within a laboratory setting. Seven chemicals tested by Iwahashi were included in this study and were reported to be generally detected with a lower sensitivity, with IC₅₀ (growth inhibition) values approximately one order of magnitude higher than the EC₅₀s reported in Table 1. Goldblum *et al.*

developed a membrane covered oxygen electrode coupled with immobilised yeast to screen for toxic chemicals based on changes in respiratory activity.²⁹ Significant disadvantages of this approach were the need to grow the cultures to specific densities and in a particular growth phase, and complicated interpretation of the results. Campanella *et al.* also immobilised yeast cells on an electrode to form a toxicity sensor. The electrode in this instance was sensitive to variations in pH as a result of carbon dioxide development during cell respiration.³⁰ Various electrode structures were examined each giving broadly similar results for a limited selection of toxicants. The assay was one and two orders of magnitude less sensitive than the assay presented here for mercury and cadmium ions respectively. Koch *et al.* proposed a yeast toxicity test as an alternative to using vertebrates in pharmaceutical screening.³¹ Yeast IC₅₀s were calculated from the influence of the test samples on growth rate and correlated to animal LD₅₀s. Repeated counting of the number of cells was required, performed either manually using a microscope or in an automated fashion using a Coulter cell counter. Such equipment is expensive and immobile, restricting its use in an environmental setting.

In comparison to previously reported cytotoxicity methods using yeast, the assay protocol presented here represents a significant simplification in protocol, instrumentation and data interpretation, whilst presenting improved sensitivity. Since cell proliferation and yield is assessed, the endpoint for cytotoxicity is broadened from a single aspect of cellular physiology to encompass the total effect on a population's ability to reproduce.

Pure compounds – genotoxicity

An example genotoxicity result is shown in Fig. 6 for nickel(II) ions. This figure demonstrates the induction response for a weaker genotoxin in the region of the threshold line. Nickel was the only metal ion detected as genotoxic in this assay. In general, carcinogenic metal compounds are only weakly mutagenic with *in vitro* test systems and are more noted for their interaction with DNA repair, enhancing the genotoxicity and cytotoxicity of other cytostatic agents.^{32,33} In addition the genotoxic potential of metal ions is highly dependent on the nature of their chemical ligand, or speciation. According to The National Toxicology Program (NTP), run by the U.S. Department of Health and Human Services, and TOXNET, run by the Toxicology and Environmental Health Information Program (TEHIP) at the US National Library of

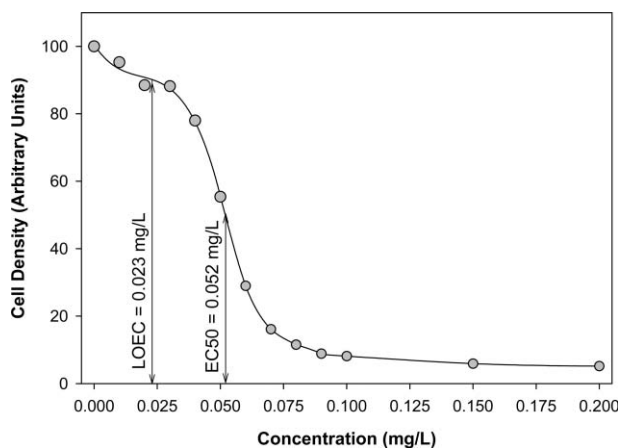


Fig. 5 Cytotoxicity profile for copper(II) ions.

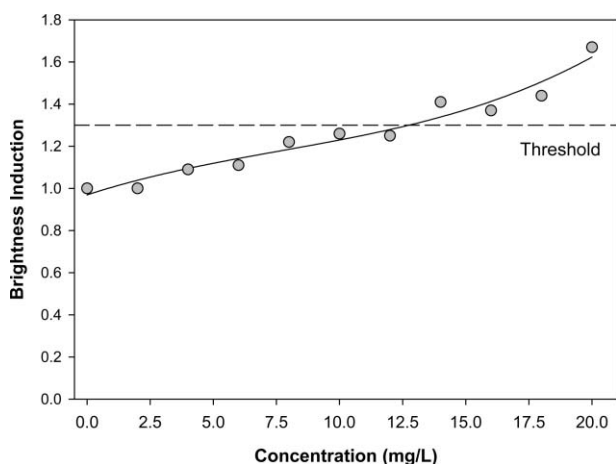


Fig. 6 Genotoxicity profile for nickel(II) ions.

Medicine, at Bethesda, MD, of the metal ions tested here only dichromate, cadmium and nickel salts are classified as “Group 1”, posing a carcinogenic risk to humans and produce positive genotoxicity results in various cell based assays. Interestingly nickel is positive in another eukaryotic assay, using mouse lymphoma cells, yet negative in the bacterial Ames test. Other studies have also indicated that when present as the sulfate ion, nickel shows a high propensity for mutagenic activity.³⁴ Dichromate, and cadmium especially, were shown to be highly cytotoxic in the yeast assay and so genotoxic responses may be masked. Since the cytotoxicity of these species is high, their acute toxicity, rather than chronic genotoxicity, is of greater relevance. A study by Codina *et al.* compared various microbial assays for the detection of genotoxicity in solutions of cadmium, chromium, copper, mercury, nickel and zinc species.³⁵ The Ames test and *E. coli* WP2 assay only detected dichromate as mutagenic, whilst the Mutatox and SOS tests detected all the metals as mutagenic. In each case however, the chloride salt was tested, and positive results were only clearly evident after 40 to 48 hours, outside the time frame of the yeast assay reported here.

A clear positive result was obtained for mechlorethamine HCl. This is the hydrogen chloride salt of a “nitrogen mustard”, a chemical warfare agent with the military designation HN2. The agent is a highly toxic vesicant and blister agent, with moderate environmental persistence.³⁶ NTP classify the chemical as an anticipated human carcinogen and positive Ames, *E. coli* and mammalian cell tests support this conclusion. Its genotoxic potential may principally derive from the strong alkylating ability of its hydrolysis breakdown products. The incubation time of the assay protocol however, (approx. 1 day) means that the assay has applications in risk management for operating in, or cleaning up, potentially contaminated sites, rather than rapid warning of the presence genotoxic agents in the event of an attack involving chemical weapon deployment.

The pesticide paraquat produced a positive genotoxicity result. This is consistent with several other studies that have identified the mutagenic potential of this compound in a range of eukaryotic systems.^{37–39} The genotoxicity of paraquat may be attributed to its ability to generate oxygen free radicals during its metabolism. Two solvents, ethanol and DMSO also gave a positive genotoxic response in the assay, although only at high test concentrations known to cause general denaturing of cellular components, membranes and proteins.⁴⁰ The concentrations in which cytotoxicity has been observed for these solvents far exceed international guidelines on the maximum test concentrations for genotoxicity. The guidelines were developed since these denaturing effects at high test concentrations often confounded the genotoxicity evaluation leading to false positive results.

Industrial effluent samples

The authors are currently participating in a BIO-WISE Demonstrator Project funded by the UK Government Department of Trade and Industry in conjunction with Gentronix Ltd. who are commercially developing this technology,⁴¹ and in collaboration with 10 chemical companies from the Specialised Organic Chemicals Sector Association (SOCSA),⁴² two biosensor suppliers (Euroclone Ltd., Wetherby, UK and Vickers Laboratories, Pudsey, UK), the Environment Agency (for England and Wales) and AstraZeneca Ltd. (project managers) based at Brixham Environmental Laboratory, Brixham, UK. The project aims to assess the potential of novel biosensors, such as that reported here, to directly detect toxicity in effluents provided by the SOCSA members. The results are to be compared with other environmental toxicity monitoring assays using algae, *Daphnia* and *Thamno* organisms.

Preliminary data from the trial is presented in Table 2 showing the results from the testing of 34 whole effluent samples. The samples were taken from a variety of UK industries including those manufacturing fine organic chemicals and inorganic pigments. The identity of the supplier remained confidential. EC₅₀ and LOEC values are quoted from the yeast assay along with an assessment of genotoxicity, following the protocol described. The results of a standard *Daphnia magna* test are also presented for comparison. Typical test concentrations of 0.1, 0.32, 1.0, 3.2 and 10% effluent were tested with the *Daphnia* screen. 10 organisms (<24 hours old from parent animals that had previously produced at least one brood of offspring) were used at each concentration tested, and maintained at 20 ± 1 °C with a photoperiod of 16 hours light, 8 hours dark and 20 minutes dawn and dusk transition periods. The reconstituted water medium used for testing was Elenđt’s M4 *Daphnia* medium.⁴³ The *Daphnia* were observed after 48 hours and organisms which were unable to move, relative to the liquid, within a period of 15 seconds, were considered immobile, even if the movement of appendages was visible. Test solutions were not aerated and the *Daphnia* were not fed during this period. Toxicity results are presented as a concentration range in which an effect is observed and an EC₅₀ predicted using Stephan’s Method.⁴⁴

Since the yeast assay is simple and rapid to set-up, a larger number and wider range of concentrations of effluent could be examined in the same time period. Effluents were tested at 2.5, 5, 10, 15, 20, 30, 40 and 50%, with 50% representing the highest concentration possible, *i.e.* 1 ml of undiluted effluent added to 1 ml of yeast reagent. Effluent dilution was made with distilled water. Where the toxicity was significant in the range 0–5%, the assay was repeated using eight, more diluted concentrations across the narrower range of interest in order to increase the accuracy of the result.

The samples presented a wide range of characteristics including the presence of particulates, organic matter, significant colour and autofluorescence, extremes of pH and contamination by organic solvents (see Table 2). In general, although the interference presented by particulate or autofluorescent contaminants was significant and readily recorded in the instrumentation, the corrective procedures described were sufficiently effective to remove this from the measurement. It was noted that it was important that the comparative control which contains no cells, should be made up with the effluent and growth media rather than water, since the sample characteristics *i.e.* colour and precipitation, could in some instances be modified by the presence of media components.

Comparing the sensitivity of cytotoxicity in the yeast assay to the *Daphnia* screen, the assays gave comparable results in 62% (21) of the effluents tested. This 62% breaks down as follows: 32% (11) of effluents were non-toxic to both yeast and *Daphnia* in the concentration ranges tested; 12% (4) were toxic in the

Table 2 Summary of cytotoxicity and genotoxicity data for a range of industrial effluents comparing the yeast assay with a standard *Daphnia* test

Sample ID	Description ^a	pH	Daphnia Screen		YEAST			Genotoxicity	
			Toxicity range (%)	EC ₅₀ (%)	EC ₅₀ (%)	LOEC (%)	Comparison of EC ₅₀ response ^{b,c}	Result	LOEC (%)
02-0225	Transparent	11.5	1.0–3.2	1.5	4.8	1.1	<<	N	—
02-0226	Br/Translucent/Particulate	8.5–9.0	1.0–3.2	1.3	3.3	0.72	=	N	—
02-0227	Br/Translucent	7.5–8.0	>10	>10	>50	17	=(NT)	N	—
02-0228	Br/Translucent	8	>10	>10	>50	27	=(NT)	P	20
02-0229	Br/Opaque/Particulate	9	0.32–1.0	0.34	11	1.5	<<	N	—
02-0252	Pale Y/Transparent	8	>10	>10	>50	>50	=(NT)	P	20
02-0253	Br/Opaque/Particulate	7	>10	>10	>50	20	=(NT)	P	10
02-0264	Bl-Bk/Opaque/Particulate	2	0.32–1.0	0.69	3.8	0.2	<<	N	—
02-0364	Bk-Pu/Translucent/Particulate	2	3.2–10	5.7	3	0.17	>>	N	—
02-0365	Br/Translucent/Particulate	9.5	<0.1	<0.1	2.5	0.15	<<	N	—
02-0366	Br-Pu/Translucent/Particulate	6.5	>10	>10	6.3	0.94	>>	N	—
03-0078	Pale Y/Opaque/Particulate	7	1.0–3.2	1.8	>50	>50	<<	P	5
03-0079	Br/Opaque/Particulate	7	<0.1	<0.1	8.9	0.44	<<	N	—
03-0080	Gr/Opaque/Bk Particulate	7	3.2–10	4.0	23	6.2	<<	N	—
03-0081	Y-Br/Opaque/Particulate/Volatile	12	0.32–1.0	0.46	1.1	0.3	=	N	—
03-0082	Bk/Opaque/Particulate	6	>10	>10	13	4.3	=(Dap > 10)	P	3
03-0146	Bl/Clear	5	0.32–1.0	0.57	0.43	0.28	=	P	0.05
03-0147	Pale Y/Clear	7.0–7.5	>10	>10	>50	>50	=(NT)	P	5
03-0148	Pale Y/Opaque	7	>10	>10	>50	>50	=(NT)	N	—
03-0149	Br-Rd/Translucent	10.5–11	<0.1	<0.1	8.8	0.9	<<	N	—
03-0150	Transparent/Particulate	6.5	>10	>10	>50	>50	=(NT)	N	—
03-0175	Transparent/Particulate	7	>10	>10	>50	31	=(NT)	N	—
03-0176	Pale Bl/Transparent	5.0–5.5	3.2–10	4.4	0.86	0.43	>>	N	—
03-0177	Transparent	9	>10	>10	>50	21	=(NT)	N	—
03-0179	Y-Br/Transparent/Particulate	7.5	>10	>10	>50	33	=(NT)	P	5
03-0204	Rd-Br/Transparent/Volatile	7	>10	>10	11	1.5	=(Dap > 10)	N	—
03-0212	Rd-Pu/Transparent/Volatile	2	3.2–10	4.6	6.0	1.1	=	N	—
03-0213	Y-Br/Transparent/Particulate	9.5	>10	>10	19	12	=(Dap > 10)	N	—
03-0214	Opaque/Particulate	7	>10	>10	44	14	=(Dap > 10)	N	—
03-0215	Y-Br/Transparent/Br Particulate	9	>10	>10	47	26	=(Dap > 10)	N	—
03-0216	Pk-Br/Opaque/Particulate	9	>10	>10	1.3	0.15	>>	N	—
03-0252	Gy-Gr/Translucent/Particulate	9	>10	>10	27	11	=(Dap > 10)	N	—
03-0253	Y-Gr/Translucent/Bk Particulate	9	>10	>10	>50	14	=(NT)	P	5
03-0338	Rd-Br/Opaque/Volatile	3	>10	>10	4.3	0.57	>>	N	—

^a Effluent Colours: Bk, Black; Bl, Blue; Br, Brown; Gr Green; Gy, Grey; Pk, Pink; Pu, Purple; Rd, Red; Y, Yellow. ^b Equivalent results: =(NT), Non-toxic in both *Daphnia* and Yeast concentration ranges tested; =(Dap > 10), Toxicity detected in Yeast above the concentration range tested in *Daphnia* screen; =, Yeast EC₅₀ in the toxic concentration range of *Daphnia* screen. ^c Differing Results: <<, Yeast test is less sensitive for toxicity compared to *Daphnia* screen; >>, Yeast test is more sensitive for toxicity compared to the *Daphnia* screen.

same concentration range; and 18% (6) produced EC₅₀s in the concentration range of 10–50% dilution with the yeast assay, outside of the concentration range tested with *Daphnia*, in which no toxicity was observed. For 15% (5) of the effluents tested, yeast was more sensitive for toxicity, and conversely for 24% (8) *Daphnia* was more sensitive for toxicity. Of these latter 8 effluents, in the majority of cases, the yeast LOEC result is close to the toxicity range of the *Daphnia* screen. Hence, based on these preliminary results, the yeast assay does appear to be an effective screen for toxicity in whole effluent samples, in this trial demonstrating equivalent or higher sensitivity to the standard *Daphnia* screen in 26 out of 34 cases. 26% (9) effluents tested positive for genotoxicity.

A more detailed comparison of various assays' sensitivity to these effluents, using results from other organisms, biosensors and toxicity kits employing dormant organisms will be published at the conclusion of the BioWise Demonstrator Project.

Conclusions

The sensitivity of the assay has proved to be broadly similar to other cell based assays in the determination of pure compounds, and demonstrates significant correlation with the standard *Daphnia* screen in the analysis of a selection of whole effluent samples, and thus the assay is proposed as applicable to the determination of toxicity in contaminated effluent and surface water samples. The assay in its current form is not sensitive enough to determine trace contaminants in

processed, *i.e.* drinking waters, especially in the case of organic compounds. This may be due to the robust nature of the yeast cell wall.^{45,46} With this in mind current work is examining the effect of genetically modifying the yeast cells to produce compromised strains with more permeable cell walls or disabled membrane transport systems, in an effort to improve the sensitivity for this particular application.

The yeast genotoxicity and cytotoxicity assay described, demonstrates many of the characteristics required for a useful environmental assay. The method described offers low cost, high portability, ease of use, no requirement for sample pre-treatment, a rapid turn-around of 1 day, and good sensitivity. Since the assay determines the total toxicity of the whole sample, it is expected that it would best be applied in a screening capacity, highlighting those samples which need to be examined further by more detailed, and generally more expensive, tests. In the case of monitoring a water course or process stream, the assay may indicate unexpected trends in levels of toxicity over time. In the case of characterisation of contaminated land, it may improve accuracy by indicating the location of areas where toxicity is especially high and where further testing should be concentrated. In this way the intention is not to provide a replacement for the regulated tests recommended by the relevant Environment Agency, rather to provide a rapid and inexpensive preview of the regulated tests, and give useful complementary data to the battery of alternative tests. As the legislation for effluent producing industries grows and a greater importance is placed on genotoxins, so the assay will also provide a means to test for

genotoxicity which is more rapid, portable and simpler to use than other comparative tests.

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References

- 1 IPPC General Sector Guidance publication of April 2001, section 1.1 on Understanding IPPC and BAT. <http://www.environment-agency.gov.uk/commondata/105385/ippcgeneral.pdf> (accessed July 2003).
- 2 Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 Establishing a framework for Community action in the field of water policy. http://europa.eu.int/eur-lex/pri/en/oj/dat/2000/l_327/l_32720001222en00010072.pdf (accessed July 2003).
- 3 EU Environment Agency Report "Environment in the European Union at the turn of the century" (1999). http://reports.eea.eu.int/92-9157-202-0/en/tab_abstract_RLR (accessed July 2003).
- 4 S. F. D'Souza, *Biosens. Bioelectron.*, 2001, **16**, 337.
- 5 P. J. O'Connell and G. G. Guilbault, *Anal. Lett.*, 2001, **34**, 1063.
- 6 C. M. Bragaglia, *Chem. Biochem. Eng. Q.*, 1998, **12**, 183.
- 7 J. L. Marty, B. Leca and T. Noguer, *Analisis*, 1998, **26**, M144.
- 8 I. Karube and Y. Nomura, *J. Mol. Catal. B: Enzymatic*, 2000, **10**, 177.
- 9 C. J. Keddy, J. C. Greene and M. A. Bonnell, *Ecotoxicol. Environ. Saf.*, 1995, **30**, 221.
- 10 B. Isomaa, H. Lilius and C. Råbergh, *ATLA-Alternatives to Laboratory Animals*, 1994, **22**, 243.
- 11 <http://www.azurenv.com> (accessed July 2003).
- 12 S. M. Steinberg, E. J. Poziomek, W. H. Engelmann and K. R. Rogers, *Chemosphere*, 1995, **30**, 2155.
- 13 F. G. Doherty, *Water Qual. Res. J. Can.*, 2001, **36**, 475.
- 14 B. T. Johnson, *Environ. Toxicol.*, 2000, **15**, 253.
- 15 T. S. C. Sun and H. M. Stahr, *J. AOAC Int.*, 1993, **76**, 893.
- 16 <http://www.thermo.com> (accessed July 2003).
- 17 D. van der Lelie, L. Regniers, B. Borremans, A. Provoost and L. Verschaeve, *Mutat. Res.: Genetic Toxicol. Environ. Mutagen.* 1997, **389** 279.
- 18 L. Verschaeve, J. van Gompel, L. Thilemans, L. Regniers, P. Vanparys and D. van der Lelie, *Environ. Mol. Mutagen.*, 1999, **33**, 240.
- 19 C. Baumstark-Khan, A. Rode, P. Rettberg and G. Horneck, *Anal. Chim. Acta*, 2001, **437**, 23.
- 20 R. M. Walmsley, N. Billinton and W.-D. Heyer, *Yeast*, 1997, **13**, 1535.
- 21 N. Billinton, M. G. Barker, C. E. Michel, A. W. Knight, N. J. Goddard, P. R. Fielden and R. M. Walmsley, *Biosens. Bioelectron.*, 1998, **13**, 831.
- 22 A. W. Knight, N. J. Goddard, P. R. Fielden, M. G. Barker, N. Billinton and R. M. Walmsley, *Meas. Sci. Technol.*, 1999, **10**, 211.
- 23 *Green Fluorescent Proteins: Proteins, Properties, Applications and Protocols*, ed. M. Chalfie and S. Kain, Wiley and Sons, Chichester, West Sussex, UK, 1st edn., 1998.
- 24 V. Afanassiev, M. Sefton, T. Anantachaiyong, M. G. Barker, R. M. Walmsley and S. Wöflfl, *Mutat. Res.: Genetic Toxicol. Environ. Mutagen.*, 2000, **464**, 297.
- 25 R. M. Walmsley, D. J. C. Gardner and S. G. Oliver, *Mol. Gen. Genet.*, 1983, **192**, 361.
- 26 D. J. B. Dalzell, S. Alte, E. Aspichueta, A. de la Sota, J. Etxebarría, M. Gutierrez, C. C. Hoffmann, D. Sales, U. Obst and N. Christofi, *Chemosphere*, 2002, **47**, 535.
- 27 Environment Canada, Web site, "Waiting for the Fiddler, Pesticides and the Environment in the Atlantic Region," Nov., 1999. (<http://199.212.16.11/epb/fiddle>) (accessed July 2003).
- 28 H. Iwahashi, K. Fujita and Y. Takahashi, *Water Sci. Technol.*, 2000, **42**, 269.
- 29 D. K. Goldblum, S. E. Holodnick, K. H. Mancy and D. E. Briggs, *Environ. Prog.*, 1991, **10**, 24.
- 30 L. Campanella, G. Favero, D. Mastofini and M. Tomassetti, *Sensors and Actuators B*, 1997, **44**, 279.
- 31 H. P. Koch, M. Hofeneder and B. Bohne, *Methods Find. Exp. Clin. Pharmacol.*, 1993, **15**, 141.
- 32 D. Beyersmann, *Toxicol. Lett.*, 1994, **72**, 333.
- 33 A. Hartwig, I. Krüger and D. Beyersmann, *Toxicol. Lett.*, 1994, **72**, 353.
- 34 G. G. Fletcher, F. E. Rossetto, J. D. Turnbull and E. Nieboer, *Environ. Health Perspect.*, 1994, **102**, 69.
- 35 J. C. Codina, C. Pérez-Torrente, A. Pérez-García, F. M. Cazorla and A. de Vicente, *Arch. Environ. Contam. Toxicol.*, 1995, **29**, 260.
- 36 N. B. Munro, S. S. Talmage, G. D. Griffin, L. C. Waters, A. P. Watson, J. F. King and V. Hauschild, *Environ. Health Perspect.*, 1999, **107**, 933.
- 37 A. Z. E. Salam, E. H. A. Hussain, H. A. Elitriby, W. A. Anwar and S. A. Mansour, *Mut. Res.*, 1993, **319**, 89.
- 38 A. C. C. Rios, D. M. F. Salvadori, S. V. Oliveira and L. R. Ribeiro, *Mutat. Res.: Fund. Mol. Mech. Mutagen.*, 1995, **328**, 113.
- 39 G. Ribas, G. Frenzilli, R. Barale and R. Marcos, *Mutat. Res.: Genetic Toxicol.*, 1995, **344**, 41.
- 40 W. Zhang, D. L. Needham, M. Coffin, A. Rooker, P. Hurban, M. M. Tanzer and J. R. Shuster, *J. Ind. Microbiol. Biotechnol.*, 2003, **30**, 57.
- 41 <http://www.biowise.org.uk> (accessed July 2003).
- 42 <http://www.socsa.org.uk> (accessed July 2003).
- 43 B. P. Elendt and W. R. Bias, *Water Res.*, 1990, **24**, 1157.
- 44 C. E. Stephan, *Methods for calculating an EC₅₀*. In: *Aquatic Toxicology and Hazard Evaluation*, Eds. F. L. Mayer and J. L. Hamelink, *Proceedings 1st Annual Symposium on Aquatic Toxicology*, ASTM, 1977, STP 634, 65.
- 45 M. Stratford, *Yeast*, 1994, **10**, 1741.
- 46 H. Zlotnik, M. P. Fernandez, B. Bowers and E. Cabib, *J. Bacteriol.*, 1984, **159**, 1018.