Session 5

Development of Diagnostic Methods for Agrochemicals

Chairman and
Session Organiser Dr S Hadfield

ANTIBODIES AND PESTICIDE ANALYSIS: CURRENT POSITION AND FUTURE PROSPECTS

M R A MORGAN, H A LEE, G M WYATT and S GARRETT
Department of Food Molecular Biochemistry, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK.

ABSTRACT

Antibody-based methods of analysis for pesticides have been increasingly used in agri-food and environmental applications over the last 10-15 years as a consequence of their advantages of sensitivity, specificity, simplicity and cost over alternative procedures. However, future advances in immunochemical technology are expected to improve analytical capability still further, leading to increased consumer assurance and efficiency of use.

INTRODUCTION

The *in vitro* use of antibodies for development of methods of analysis found widespread application in the clinical field following the first description of modern immunoassay in 1959 (Yalow & Berson, 1959) and further application to compounds of low molecular weight 8 years later (Beiser & Erlanger, 1967). Antibodies are able to target a molecule with high affinity and specificity, as you would expect given their *in vivo* function. Interestingly, scientists had long been able to stimulate antibody production towards 'artificial' targets, and many decades earlier had fully described the principles governing antibody specificity towards low molecular weight compounds (Landsteiner, 1945).

The high affinity of antibodies enabled analytical methods of considerable sensitivity to be generated; the high specificity allowed sample preparation to be kept to a minimum. The use of batch processing of samples gave high sample through-puts. That assay costs were often comparatively low, was usually secondary to the fact that immunoassays could generate results where alternative procedures were unable to be applied. At the present time, even though instrumentally-based methods of analysis have improved considerably, there are still examples of analytes that can only be tackled using an antibody - including determinations of wheat gluten and its quality (Mills *et al.*, 1995).

Immunoassays are now being applied well beyond the clinical area into agri-food and environmental analysis, a development that was considerably enhanced with the description of non-isotopic labels to follow the course of the antibody-target interaction (van Weeman & Schurrs, 1971; Engvall & Perlman, 1971). Now the diversity of immunoassay formats is one of its strengths, ranging from quantitative to semi-quantitative, from laboratory to field (and even home) use, from single sample to batch processing with full automation, from use by skilled personnel to the totally untrained. It is, however, important to note that all immunoassays are

based on the Law of Mass Action, even though the antibody at the centre of the assay is of biological origin, and are capable of being fully quantitative.

ANTIBODY PRODUCTION FOR RECOGNITION OF PESTICIDES

Antibodies are produced in higher animals in response to challenge by cells and molecules recognised as 'non-self' and 'foreign'. The other limitation is that molecules need to possess a minimum molecular weight of between (roughly) 1,000 and 10,000 daltons. Production of antibodies against molecules of the necessary size is, therefore, a case of selecting the appropriate animal and beginning an immunisation programme designed to yield the desired antibodies in the appropriate amounts, taking into account considerations of specificity as necessary.

Fortunately, it is also possible to produce antibodies to low molecular weight molecules - known as haptens - by covalently linking them to a carrier, 'foreign' molecule and proceeding as before. The chemistry of the conjugation is critical to the outcome of the immunisation as far as specificity is concerned; greatest specificity being observed for those portions of the molecule distal to the site of linkage. Some of the antibodies raised to the whole are capable of recognising the hapten alone. It is with such antibodies that we are primarily concerned with in order to analyse pesticides. For further details of antibody production, the reader is referred to one of the many specialised texts available.

Polyclonal or monoclonal antibodies?

Many antibodies are produced by animals in response to challenge, each capable of recognition with a different affinity and specificity. Each of the different antibodies is produced by a different line of cells (or clones). Hence the immune serum contains many antibodies and is a polyclonal antiserum. Kohler & Milstein (1975) described a procedure for production of single populations of antibodies, each arising from single clones, and known as monoclonal antibodies. It remains a widespread misconception that monoclonal antibodies are superior to polyclonal antibodies. Whilst it is true that production of monoclonal antibodies *in vitro* offers the potential for production of (theoretically) unlimited amounts of antibody, it is also true that the high titres and high volumes of a good polyclonal serum can offer effectively unlimited quantities. The key factor in an anti-pesticide antibody is its properties:

(i) does it have the right specificity? The chemistry of hapten-protein conjugation is the most important factor, not the nature of the antibody.

(ii) does it have the right affinity? The dominant antibody in a polyclonal preparation at high dilution will be a high affinity antibody. Unless special precautions are taken, a monoclonal antibody will be of average affinity.

(iii) will it have the right properties for interaction with sample matrix or extraction solvents? In theory, it should be possible to select monoclonal antibodies for desirable properties such as the ability to tolerate high levels of particular solvents or matrix co-extractants, but this is, unfortunately, hardly ever done in practice. A polyclonal antiserum can just as easily have the right properties.

The only occasions when it would be essential to be thinking of a monoclonal antibody would be for commercial applications requiring large quantities of antibodies. Examples include the production of immuno-affinity columns.

FORMATS FOR IMMUNOASSAY OF PESTICIDES

As stated earlier, there are now a wide range of formats available and selection of any one will be determined by the requirements of each application and by the availability of proprietary procedures from the immuno-diagnostic industry. Of most relevance to users (apart from the availability of the desired antibodies) will be factors relating to the equipment required, the time taken for the analysis, whether quantitative or semi-quantitative, how many samples can be handled, or where the analysis can be performed. Issues of whether the assay is to be performed on microtitration plates, in tubes or with magnetic beads are less of a factor. The ability of an immunoassay, in whatever format, to deal with a particular type of sample matrix ought to a prime consideration in choosing that assay. The presence or absence of non-specific interference will be antibody-dependent first and foremost, with format a secondary contributor. However, the diversity of potential sample matrices means that results are often limited to one or two applications. One area where sections of the immuno-diagnostic industry could provide clearer information relates to the matrices for which a kit has been validated; often minimal information is provided, occasionally mis-leading.

Sample selection and preparation can take more time (even for an immunoassay) than the assay itself. Indeed, it could be argued that even more time should be devoted to sample selection, given that it is the single most important factor in determining the significance of the analytical result even above the performance of the assay itself. It could be said that not enough time is given to linking performance of the assay with efficient sample selection and preparation. Examples of different types of formats being applied to pesticide analysis include the use of magnetic particles (Rubio *et al.*, 1991), flow injection immunoanalysis (Dietrich & Kramer, 1995) and microtitration plates (Wittman & Hock, 1990; Lucas *et al.*, 1995)

PESTICIDE IMMUNOASSAYS: CURRENT SITUATION

The advantages of immunoassays mean that they are now an important part of the repertoire of the pesticide analyst. From a position where immunoassays were first used as a cheap 'look see' procedure, they have developed considerably and are now used in all aspects of pesticide development, registration and application, and in monitoring of agri-food and environmental samples. There is an active diagnostics industry supporting the activities of researchers developing new antibodies, new formats and new applications of the technique. There is an extensive literature on pesticide immunoassays, and as an introduction those readers new to the area two recent texts will provide a suitable starting point (Kurtz *et al.*, 1995, Nelson *et al.*, 1995).

The limiting factors on further development and application of the technique are the availability of antibodies of the most appropriate properties. Production of an antibody against a haptenic

molecule can take a minimum of 6 months, but more often up to 2 years (whether a polyclonal or monoclonal antibody is sought). The process will usually require sophisticated chemistry, much experience of anti-hapten antibody production and screening techniques, and luck.

APPLICATION OF CURRENT RESEARCH TO PESTICIDE IMMUNOASSAYS

There are several areas of research addressing problems of antibody production, and these will be discussed briefly in turn. The list is not exclusive, but covers those of most likely general relevance.

Molecular modelling of hapten structures

It has been almost a tradition amongst immunochemists in years past to gloss over and race through the essential step of hapten-protein conjugation. There have been two reasons for this, relating firstly to the highly variable nature of the immune response which has in some cases compensated for chemistry of a rather dubious nature, and secondly to the difficulties inherent in fully characterising a protein conjugate once made. This era is now over. The properties of the antibodies now required leave no room for a lack of precision at the critical stage of immunogen production. Design must take into account factors such as the ultimate specificity required, the metabolism and stability properties of the pesticide. One step forward has been the use of molecular modelling to gain a more advanced insight into the real structure of the hapten as presented to the immune system. Both space-filling and charge distribution can be modelled, and a fuller understanding of the dynamic situation envisaged. Such work has allowed the explanation of apparently anomalous cross-reactions for haptens (Elissalde *et al.*, 1995), and is starting to be used in a predictive rather than reactive role (Spinks *et al.*, 1995). It is envisaged that molecular modelling for hapten design will increase in use.

Recombinant antibodies

Some years ago, it was reported that new procedures based on molecular biology would revolutionise antibody production, allowing identification of an antibody within days, making possible the manipulation and improvement of antibody properties, and avoiding the need for immunisation (Ward *et al.*, 1989). The potential advantages provided by the use of recombinant antibodies are clear, but the reality has been rather slower in arriving. Recombinant antibodies against pesticides are now being described (Bell *et al.*, 1995; Garret *et al.*, 1995; Byrne *et al.*, 1990; Kramer & Hock, 1996)), though at present the main advantages seem to lie in the potential for manipulation of antibody properties.

Antibody structure is now well understood in general terms through the use of X-ray crystallography. Indeed, computer programmes are now available for modelling of individual antibody structure through a knowledge of the sequence. Further, claims are made for programmes able to model interaction of the antibody with the target. In practice, considerable expertise is still required for even comparatively simple systems; for haptens, the situation is more complex since our understanding of antibody-target interactions is almost solely based on antibody-protein interactions.

Manipulation of antibody structure towards defined ends can occur at two extremes - random mutagenesis, and rational design. Bearing in mind that with haptens we are already at an extreme of antibody interaction (and, of course, not part of any normal *in vivo* response) truly rational design will have some years to wait. We have recently begun a programme of manipulation of the structure of an anti-parathion antibody fragment expressed in E. coli (Garrett *et al.*, 1995; Wyatt *et al.*, 1996). It is clear that with anti-hapten antibodies, strategies will have to be developed that go beyond manipulation of the CDR3 of the heavy chain alone. Given that many haptens will have sizes close to that of the amino acid components of the antibody, it is not surprising that a subtle approach will be called for.

Anti-idiotype antibodies

Antibodies can be raised against antibodies, and, in particular, raised against the binding sites of those antibodies. Such second generation antibodies are known as anti-idiotype antibodies, and are of interest because they can share properties with the original target of the primary antibody. One can go further and generate a third generation (anti-anti-idiotypes) and mimic the original antibody.

The reasons for doing such seemingly complex manipulations usually relate to an interest in the additional possibilities provided of devising new assay formats. Though such research has not yet generated commercial diagnostic spin-offs, it does seem a possibility for the future. Spinks *et al.*, (1993) were interested in the multi-analyte approach of Ekins *et al.*, (1990). Hsu *et al.*, (1995) have described the use of anti-anti-idiotype antibodies. Barnard *et al.*, (1990) have described a format which will give a standard curve for a hapten that directly relates signal to mass, a considerable step forward.

Biosensors

Biosensors capable of real-time measurements have long been a goal of analysts. A particular attraction is the monitoring of environmental samples, such as waste or river water. Though considerable advances have been made, the final breakthrough required for sensitive detection of potential environmental contaminants in real time remains elusive.

SUMMARY

The use of antibody-based methods of analysis have bought considerable benefits to pesticide analysts. Continued research, particularly on methods of antibody production, offers the potential for additional progress in the future. Such progress will benefit pesticide producers, users, regulatory agencies and consumers alike.

REFERENCES

Barnard, G; Kohen, F; (1990) Idiometric assay: noncompetitive immunoassay for small molecules typified by the measurement of estradiol in serum. *Clinical Chemistry*, **36**, 1945-1950.

Beiser, S M; Erlanger, B F (1967) Estimation of steroids by an immunochemical technique. *Nature*, **214**, 1043-1044.

Bell, C W; Roberts, V A; Scholthof, K-B G; Zhan, G; Karu, A E (1995) Recombinant antibodies to diuron: a model for the phenylurea combining site. In: Immunoanalysis of agrochemicals. Emerging Technologies. *ACS Symposium Series 586*, ACS Washington, DC, USA.

Byrne, F R; Grant, S D; Proter, A J; Harris, W J (1996) Cloning, expression and characterisation of a single-chain antibody specific for the herbicide atrazine. *Food and Agricultural Immunology,* in press.

Deitrich, M; Kramer, P M (1995) Continuous immunochemical determination of pesticides via flow injection immunoalaysis using monoclonal antibodies against terbutryne immobilised to solid supports. *Food and Agricultural Immunology.* **7,** 203-220.

Ekins, R; Chu, F; Biggart, E (1990) Multispot, multianalyte, immunoassay. *Annales de Biologie Clinique,* **48**, 655-666.

Elissalde, M H; Kamps-Holtzapple, C; Beier, R; Plattner, R D.; Rowe, L D; Stanker, L H (1995). Development of an improved monoclonal antibody-based ELISA for fumonisin B_{1-3} and the use of molecular modelling to explain observed detection limits. *Food and Agricultural Immunology,* **7,** 109-122.

Engvall, E; Perlman, P (1971) Enzyme-linked immunosorbent assay (ELISA) Quantitative assay of immunoglobulin G. *Immunochemistry,* **8,** 871-874.

Garrett, S; Wyatt, G; Lee, H A; Morgan, M R A (1995). Sequence analysis and expression of single-chain variable fragment antibodies generated from a parathion-specific monoclonal antibody. *Abstracts of 1995 International Chemical Congress of Pacific Basin Societies.* Honolulu, Hawaii, USA.

Hsu, K-H; Chu, F S (1995) Anti-idiotype and anti-anti-idiotype antibodies for aflatoxin from laying hens. *Food and Agricultural Immunology,* **7**, 163-174.

Kramer, K; Hock B (1996) Recombinant single chain-antibodies against s-triazines. *Food and Agricultural Immunology*, in press.

Kohler, G; Milstein, C (1975) Continuous culture of fused cells secreting antibody of defined specificity. *Nature*, **256**, 495-497.

Kurtz, D A; Skerritt, J H; Stanker, L; (1995). New frontiers in agrochemical immunoassay. *AOAC International,* Arlington, VA, USA.

Landsteiner, K (1945) In: *The specificity of serological reactions*. Harvard University Press, Boston, USA.

Lucas, A D; Goodrow, M H; Seiber, J N; Hammock, B D (1995). Development of an ELISA for the N-dealkylated S-triazines: application to environmental and biological samples. *Food and Agricultural Immunology,* **7,** 227-242.

Mills, E N C; Brett, G M; Holden, S; Kauffman, J A; Tatton, M J; Morgan, M R A; (1995) Production of monoclonal antibodies to gluten proteins and their use in developing tests for gluten quality. *Food and Agricultural Immunology*, **7**, 189-196.

Nelson, J O; Karu, A E; Wong, R. B (1995) Immunoanalysis of agrochemicals. Emerging Technologies. *ACS Symposium Series 586*, ACS Washington, DC, USA.

Rubio, F M; Itak, J A; Scutellaro, A M; Selisker, M Y; Herzog, D P (1991). Performance characteristics of a novel magnetic-article-based enzyme-linked immunosorbent assay for the quantitative analysis of atrazine and related triazines in water samples. *Food and Agricultural Immunology.* **3,** 113-126.

Spinks, C A; Wang, B; Mills, E N C; Morgan, M R A (1993) Production and characterisation of monoclonal anti-idiotype antibody mimics for the pyrethroid insecticides and the herbicide paraquat. *Food and Agricultural Immunology*, **5,** 13-25.

Spinks, C; Lee H A; Morgan, M R A. (1995) Molecular modelling of haptenic sulphonamides: a bridge too far. *Abstracts of 1995 International Chemical Congress of Pacific Basin Societies*. Honolulu, Hawaii, USA.

Van Weeman, B K; Schuurs, A H W M; (1974). Immunoassay using antigen enzyme conjugates. *FEBS Letters,* **15**, 232-236.

Ward, E S; Gussow, D; Griffiths, A D; Jones, P T; Winter, G (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli. Nature,* **341,** 544-546.

Wittman, C; Hock B (1990) Evaluation and performance characteristics of a novel ELISA for the quantitative analysis of atrazine in water, plants and soil. *Food and Agricultural Immunology,* **2,** 65-74.

Wyatt, G M; Garrett, S; Lee, H A; Morgan, M R A (1996) Alteration of binding properties of an anti-parathion recombinant antibody by mutagenesis of the CDR3 region of the VH domain, Submitted manuscript.

Yalow, R; Berson, S A (1959) Assay of plasma insulin in human subjects by immunological methods. *Nature*, **184**, 1648-1649

LABORATORY APPLICATIONS OF IMMUNOASSAYS FOR PESTICIDE RESIDUE DETECTION

R MAYCOCK
DowElanco Europe, Letcombe Laboratory, Letcombe Regis, Letcombe, Wantage, Oxon, OX12 9JT, UK

ABSTRACT

This review will focus upon the requirement of laboratories to generate and evaluate data specifically for the study of pesticides and their residues. The presentation will consider the range of classical analytical techniques available and compare their benefits and limitations with those from the new generation of immunological-based tests. The general criteria which will determine the acceptability of various test methods will clearly depend upon the final purpose to which they will be targeted e.g. the provision of regulatory data. In order to make decisions regarding the value of immunoassays, this presentation will use case study examples. First, a generic pesticide immunoassay for the spinosad insecticide group in a range of environments including soil, water and crops will be described followed by the detection of the herbicide triclopyr in soil-water environments. Overall, this review will illustrate the type of data produced and the opportunitities to use novel diagnostic methods for laboratory assessment of pesticides and their residues.

A NEW DIAGNOSTIC METHOD MORE ACCURATE AND PRECISE THAN GC-MS

C R LOWE
Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QT

L J COX, S C WILLIAMS, A M PELOE, S H L CARTWRIGHT, S S EAPEN, K A LEMON, J M McCANN
Environmental Sensors Ltd, Downhams House, Downhams Lane, Cambridge, CB4 1XT

ABSTRACT

We have developed a novel chemiluminescence immunoassay method ("Lumia™) for pesticide detection that is now being used for regulatory testing of pesticides by the UK water authorities. The method is rapid, low cost, accurate and highly automated. Moreover, as a result of high sensitivity and improved recoveries, it offers the user results better than those obtainable from gc-ms. The method is therefore highly relevant to the needs of the agrochemicals industry for product development and environmental fate testing. Further development of the method is being carried out to produce a hand held "dipstick" version for field use.

INTRODUCTION

The agrochemicals industry is being challenged by ever greater regulatory hurdles in terms of both development and end use of its products. This has resulted in increased analytical testing for residual levels of pesticides both by the agrochemical companies themselves and among outside regulators and users, such as food manufacturers, the water industry, national agencies, regional governments and municipalities. Traditional methods of analysis, such as gc-ms, are designed for relatively low numbers of samples and use by skilled technicians within a sophisticated laboratory environment.

In response to the regulatory problems of the water industry, which is faced with stringent EC regulations regarding the pesticide content of drinking water, we have developed a new detection system for pesticides using chemiluminescence immunoassay. The system is designed to perform as well as the existing analytical methods at a fraction of the cost. Indeed, the method is now being used both for regulatory compliance and operational testing of pesticides by a major UK water PLC. Since the data from the system are as good as or better than gc-ms it offers the agrochemicals industry:

- Improved environmental fate and regulatory testing during product development at lower cost.

- A low cost, sensitive and accurate analytical method that is available to users and regulators to speed product acceptance and use.

Additional development of the system is being carried out to produce a "dipstick" method suitable for field use.

THE CHEMILUMINESCENCE SYSTEM

The chemiluminescence system applies the latest technology to pesticide detection at very low levels. The method relies upon the competition between pesticide in the sample and labelled pesticide for a limited number of antibody binding sites. The greater the amount of pesticide in the sample, the fewer antibodies are available for binding to the labelled pesticide; as a consequence the analytical signal falls. The inverse relationship between the amount of pesticide in the sample and the analytical signal can be used for quantitation of the pesticide concentration.

Instead of a colorimetric label, the chemiluminescence system uses a more sensitive luminescence label that is now the method of choice for high sensitivity measurement in the healthcare industry. This is combined with high precision automated instrumentation to produce a system with better performance than gc-ms.

The method can be applied to any small molecule for which it is possible to produce an antibody; in consequence the system is being extended to include triazines, acid herbicides, urons and other compounds of interest to the water industry. The method will also distinguish between individual isomers.

Figure 1 describes the chemistry behind the method. Raw samples are pipetted onto a 96 well plate where they are incubated for 30 minutes for screening results or 90 minutes for high sensitivity results. The plate is then automatically washed, the luminescence labels are injected and the results processed. Processing 40 samples takes 120 minutes, about 15 minutes of which is hands on time. The results can be downloaded directly to a laboratory information system. The entire operation is automatic and training an operative takes half a day. A single system is easily capable of running several hundred samples per day.

Figure 2 shows the standard curve for the system with the characteristic inverse realtionship between signal output and concentration of analyte (atrazine in this case). Note the ability of the system to detect atrazine at very low levels.

Table 1 describes the performance of the system at the laboratories of Anglian Water PLC, the UK water company that is responsible for some of the most intensively farmed parts of the UK. The trial was carried out under the guidelines of the Drinking Water Inspectorate

(DWI) and examined the precision, accuracy, limit of detection (defined as 4.65 times the standard deviation of the zero signal) and recovery (the ability of the system to detect additions of pesticide to a sample).

The first two columns give the water source and type. The third column gives the level of herbicide (atrazine) in the sample - this was spiked up to the level required by the trial protocol.

The fourth column gives the total standard deviation between and within analytical batches and is therefore a measure of the total imprecision in the system. The target level in the regulations is the higher of 2.5 ng/l or 5% of the reading. A PASS in the column indicates that the system met these levels; the figures in brackets indicate where the system failed to meet these levels. Because the gc-ms methods fail to meet these criteria, Anglian have instituted internal criteria given in the next column of 5% or 5 ng/l. Only two marginal failures are seen in this column.

Columns 6 and 7 give limit of detection data defined as 4.65 times the standard deviation at the zero signal. Column 6 gives the DWI levels. Column 7 Anglian Water's internal levels. No failures are seen on limit of detection.

Column 8 refers to quality control standards.

Column 9 refers to spiking recovery data. The method meets the DWI criteria of 95-105%

It is important to realise that the traditional gc-ms method not only fails to met the DWI guideline levels but often fails to meet the internal Anglian levels. The performance of the system was adjudged to be superior to gc-ms and the system has now been purchased and implemented by Anglian for their central trace organics laboratory.

Table 2 describes the effect of extreme levels of interferences normally seen in water samples.

Table 3 describes cross-reactivities of other pesticides in the system. The large cross reactivity with propazine is not an issue in the EC since propazine use is banned. The main cross reactant seen is simazine which is widely used instead of atrazine. In the absence of atrazine, a cross reactivity of 4% is seen; however if the two are applied together as in real samples the cross reactivity, even with high levels of simazine, is negligible.

Table 4 compares the performance of the system to the existing gc-ms systems for atrazine determination. The analytical advantages of the system arise from two factors. First, the innate sensitivity of the method. Indeed, although the limit of detection for current methods in the water industry is 20 ng/l, we have constructed systems with considerably greater sensitivity. For the agrochemicals industry such sensitivity would allow detection of residual amounts at very low levels or the opportunity to dilute samples several times over to reduce the effect of interferents.

The second analytical advantage arises from improved sample recoveries. Since the system uses raw samples, no preconcentration or solvent recovery is needed, thereby saving time, reducing the need for additional processing and improving accuracy. In the water industry typical recoveries are in the region of 80-120% for gc-ms methods versus 95-105% for the new method.

In conclusion, the chemiluminescence technology represents the limit to which existing immunoassay technology can be pushed in terms of limit of detection, precision and accuracy. The quality of the data demands that immunoassay should therefore not only be thought of as a screening method but also as a robust analytical tool. For the agrochemical industry it should therefore be carefully considered alongside traditional methods in terms of quality of data in addition to speed, low cost and ability to deal with prodigious numbers of samples.

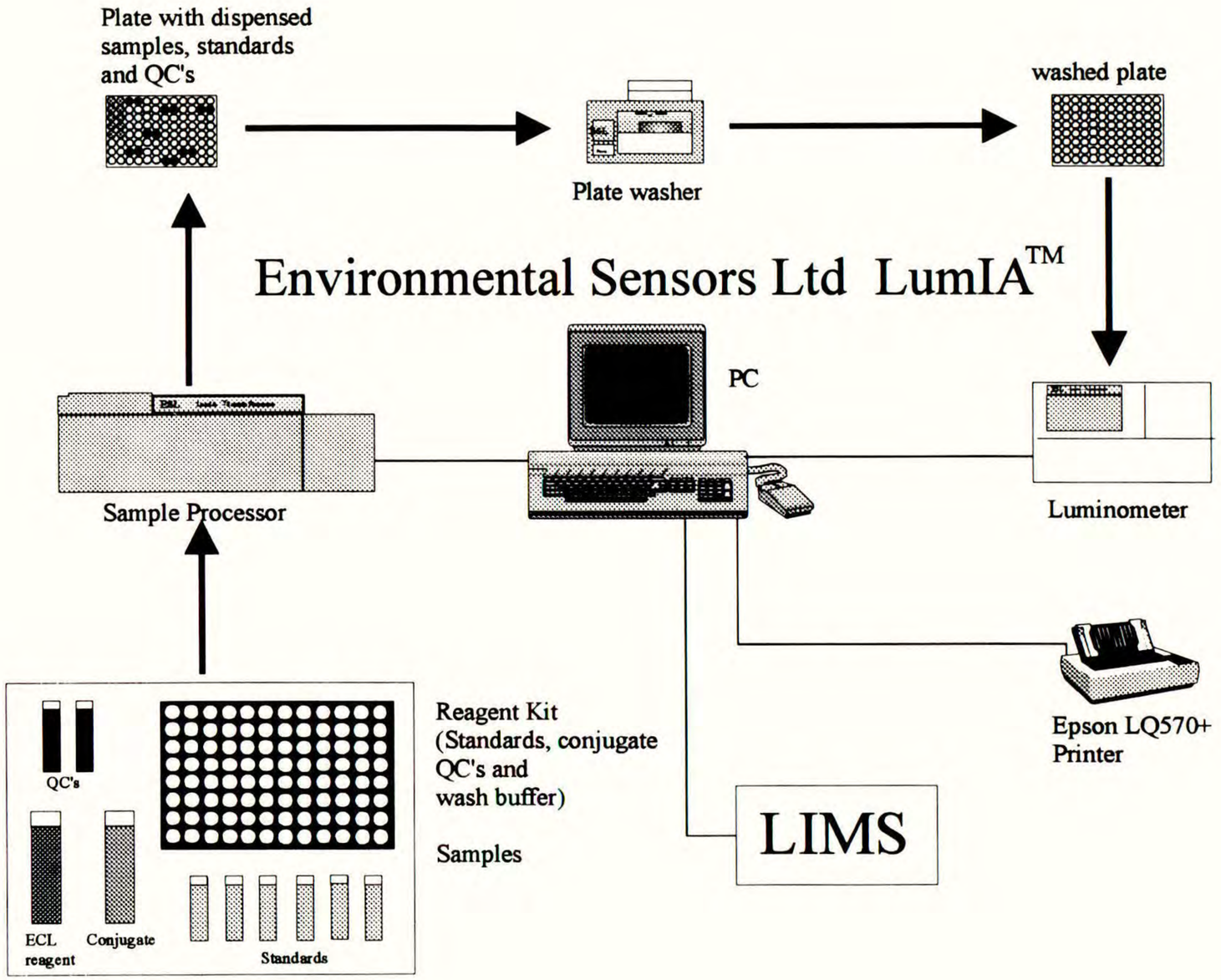

Figure 1 : Format of the Enhanced Chemiluminescence Immunoassay Instrumentation

Table 1. Performance of the LumIA™ chemiluminescence immunoassay at Anglian Waters Trace Organics Laboratory.

Anglian Water Services Ltd implemented the assay for operational control purposes in November 1995.

WATER SOURCE	WATER TYPE	SPIKED [ANALYTE] (ng/l)	PRECISION S_t< 5% or 2.5 ng/l	PRECISION S_t< 5% or 5 ng/l	LOD < 10 ng/l	LOD < 20 ng/l	CALIBRATION BIAS < 10 % or 5 ng/l	SPIKING RECOVERY 95 - 105%
NATURAL SURFACE	RAW	10 100 140	(4 ng/l) PASS (12 ng/l)	PASS PASS (12 ng/l)	(14 ng/l)	PASS	N/A	PASS
NATURAL SURFACE	TREATED	10 100 140	(5 ng/l) (10 ng/l) PASS	PASS (10 ng/l) PASS	(11 ng/l)	PASS	N/A	PASS
NATURAL BORE	RAW	10 100 140	(4 ng/l) PASS PASS	PASS PASS PASS	PASS	PASS	N/A	PASS
NATURAL BORE	TREATED	10 100 140	(4 ng/l) PASS PASS	PASS PASS PASS	PASS	PASS	N/A	PASS
STANDARDS	HPLC WATER BASED	10 - 140	PASS	PASS	PASS	PASS	PASS	N/A
KIT ANALYTICAL QUALITY CONTROLS	NATURAL WATERS	60 100	PASS	PASS	N/A	N/A	N/A	N/A

TRIAL PROTOCOL (see next page)

TRIAL PROTOCOL

The protocol for the trial was constructed in accordance with the current DWI guidelines for the assessment of analytical systems as detailed in references 1 and 2.

Four naturally occurring samples (surface raw, surface treated, bore hole raw and bore hole treated) and a series of hplc grade water samples, were spiked with known amounts of atrazine immediately prior to analysis using LumIA™ instrumentation installed at Anglian Waters Trace Organics Laboratory in Milton Keynes. Samples were run in duplicate in each batch; a total of 11 analytical batches were run, with a maximum of 2 per day, to afford a relevant number of degrees of freedom for statistical analysis.

Statistical analysis of the data was conducted in accordance with the guidelines recommended by the Water Research Centre, as detailed in reference 3.

DWI GUIDELINES :

Precision :	Total standard deviation should not exceed (at 95% confidence limits) 5% of [analyte] or 2.5 ng/l, whichever is the greater.
Accuracy:	Systematic error or bias, should not exceed 10% of [analyte] or 5 ng/l, whichever is the greater.
Limit of Detection :	Should be equal to or less than 10 ng/l (4.65 x S_w). For trace organics analysis, DWI currently regard 20 ng/l as acceptable.
Recovery :	Recovery of added analyte should be between 95 - 105 % of that added.

REFERENCES

1. Guidance on Safeguarding the Quality of Public Water Supplies. HMSO, 1989 (ISBN 0-11-752262-7).

2. Further Guidance on Analytical Systems. DWI Information Letter 8/93, 1993.

3. A Manual on Analytical Quality Assurance for the Water Industry: NS30. Water Research Centre, 1989. (ISBN 0 902156 85 3).

Figure 2: Standard Curve for Atrazine

y axis gives the response of the system in relative light units
x axis gives the concentration of atrazine in ng/l

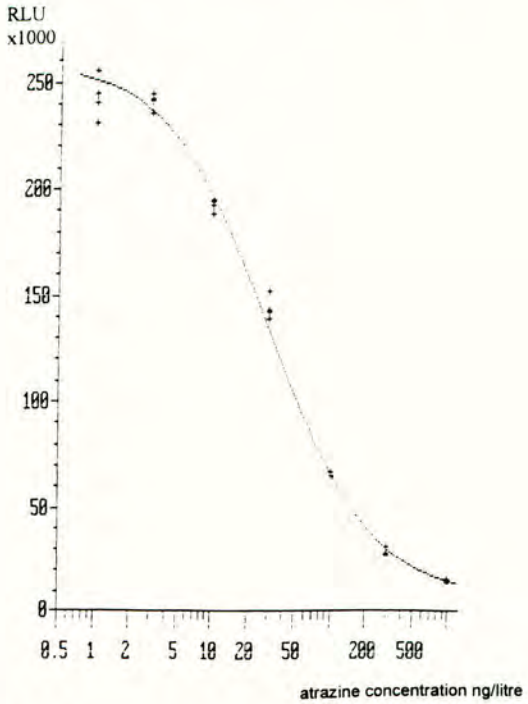

Table 2: Chemiluminescence System Interferences

The following substances do not interfere at the concentrations stated:

Interferent	Concentration
NaCl	50mg/l
HNO_3	0.02% v/v
cadmium, nickel, copper, lead ions as nitrates	1mg/l
zinc ions as nitrate	5mg/l
iron, aluminum as chlorides	1mg/l
calcium chloride	200mg/l

Table 3: Chemiluminescence System: Cross-reactivity of Other Pesticides

In the absence of atrazine

Pesticide	Percentage cross reactivity at the mid point (I_{50})of the standard curve
propazine	80%
simazine	4%
prometryn	5%
hydroxyatrazine	2%
desethylatrazine	7%

There is no cross reactivity with desisopropylatrazine, isoproturon, diuron, linuron, mecoprop, MCPA, MCPB and 2,4 D.

In the presence of atrazine at 45 ng/l, simazine at 56 ng/l produces no statisticially significant cross reactivity.

Table 4: Comparison of GC-MS with the System

	Chemiluminescence System	GC-Mass Spec
Limit of Detection ng/l	10-20	20
Preconcentration	none	yes
Extraction	none	yes
Time for run	30-120 minutes for 40 samples	45 minutes plus extraction time for 1 sample
Sample recovery	95-105%	80-120%
Cost of fully automatic system	£39,000	£60,000+
Cost per test	£10	£25-40 for screen
Operator	Limited lab training	Skilled chemist
Sample size	25 microlitres	0.5l

IMMUNOCHEMICAL TESTS TO MONITOR HUMAN EXPOSURE TO PESTICIDES: SALIVA AS SAMPLE SOURCE

B S FERGUSON
ImmunoSystems Incorporated, Div. of Millipore Corporation, 4 Washington Avenue, Scarborough, Maine 04074

H N NIGG
University of Florida, 700 Experimental Station Road, Lake Alfred, Florida 33850

ABSTRACT

In the past decade, numerous immunochemical test methods have been developed for the monitoring and measurement of a wide variety of insecticides, herbicides and fungicides. Most of these immunoassays have taken the form of *Competitive Inhibition Enzyme ImmunoAssays*, or "EIA's", and the vast majority of applications have been for the analysis of pesticides in food, water and soil matrices. A limited, though growing number, of immunochemical methods have been applied to human exposure monitoring; nearly all of these applications use urine as the sample medium. On the other hand, saliva is a universal biological fluid, painless to collect and surprisingly plentiful: the average daily secretion in humans is 500 - 1500 ml. Yet saliva has not been very well explored as a medium to monitor pesticide exposure. Our work to date has focused on fortifying saliva with such residues as parathion methyl and chlorpyrifos. Recoveries by EIA are excellent; for example, six saliva samples fortified with chlorpyrifos at 1.0 ppb yielded an average recovery of 91%. In the spring of 1996, saliva from Florida pesticide applicators will be collected pre- and post-spraying and analyzed by EIA. Immunoassay precision and reproducibility studies will be conducted and correlated with results from hplc or glc analyses, if available.

INTRODUCTION

Protecting workers from the harmful effects of pesticides requires knowlege of the dose, particularly the *internal dose*. The "dose" is a basic tenet in toxicology, perhaps most famously expressed by Paracelsus.

> What is there, that is not poison? All things are poison and nothing (is) without poison. Solely the dose determines that a thing is not poison.
>
> Paracelsus, 1492-1541
> (Deichmann et al., 1986)

For pesticides and other chemicals, there are few techniques to determine internal dose. Unfortunately, present methods typically require a well-trained technical staff and expensive equipment. The drawbacks of monitoring bodily fluids for dose estimation have been reviewed (Nigg & Stamper, 1989).
Dose estimation methods fall broadly into two categories: *direct* measurements in a body fluid, or the *indirect* measurement of a change in a physiological function. Direct measurement methods are almost exclusively limited to urine. Measurement of a pesticide or its metabolite(s) in urine is complicated analytically. The principal drawback to urinary estimation of internal dose is the lack of knowledge about human excretory pathways. The pathways are known, of course, but it is not known how the excretion of a chemical is balanced between pathways. For instance, pesticide metabolites may be excreted in sweat (Rosenberg *et al.*, 1985). In hot working conditions, humans respond by sweating more and urinating less. The human urinary excretion pathway is usually incomplete even for "biodegradable" chemicals (Nigg & Stamper, 1989). As an example, i.v.-administered 2,4-D has a 90% excretion time of 58 h (range 53-64 h) in humans. Yet malathion, used in many large eradication programs, has a 90% excretion time of 241 h (range 124-4264 h) (Nigg & Stamper, 1989).

The best example of an indirect method is the measurement of blood plasma cholinesterase and red blood cell acetylcholinesterase. The measurement of a physiological effect, like plasma cholinesterase levels, does not relate to dose. Plasma cholinesterase is approximately 10-fold more sensitive to organophosphate inhibitors than RBC acetylcholinesterase (Grob & Harvey, 1949). In addition, plasma cholinesterase has human phenotypes which are somewhat resistant to carbamate and many other inhibitors (Harris & Whittaker, 1962).

Saliva as a sampling medium may obviate all of these difficulties. Saliva is non-invasively collected, is a reflection of the free chemical in blood plasma and is a simple and logical choice for analysis because the parent compound is secreted in saliva (Nigg & Wade, 1992). The saliva concentration of a pesticide is consequently the direct indication of the amount of that chemical available to interact with tissues (Nigg & Wade, 1992). By knowing the relationship between free compound in the blood and saliva concentration, and the ratio of bound to free compound in blood, the *internal* dose at the time of sampling becomes a simple calculation. Combined with the sensitivity, specificity, portability and cost-effectiveness of immunoassay, a saliva sample can potentially provide a simple, convenient and accurate method for estimating the internal chemical dose in humans.

MATERIALS AND METHODS

Chlorpyrifos (CPE) and parathion methyl standards of analytical grade were purchased from Chem Service (West Chester, PA). The polystyrene test tubes and 96-well microELISA strips were pre-coated with polyclonal antibodies to chlorpyrifos and parathion methyl, produced in rabbits by

immunizing subcutaneously and bleeding intravenously monthly. The immunogens were prepared using bovine serum albumin conjugated to analogues of these pesticides through their phosphorothioate constituents. The peroxidase tracers were prepared to an ethyl analogue in the case of parathion methyl and using a triclopyr analogue in the case of chlorpyrifos. In both instances, the linkage was also at the phosphorothioate end of the molecules. The antibody-coated tubes and microwell plates, appropriate horseradish peroxidase (HRP) enzyme conjugate, tetramethylbenzidine (TMB) substrate and 1N HCl "stop" solutions were pre-packaged as part of EnviroGard® Chlorpyrifos (Plate) and Parathion (Tube) Kits (Millipore Corporation, Bedford, MA).

Saliva samples were collected from presumably unexposed laboratory volunteers by expectoration in glass scintillation vials. Samples were tested immediately by immunoassay or stored no longer than overnight at 4-8°C before testing.

High purity reverse osmosis (RO) water was provided by a Milli-RO *PLUS* 10 water purification system (Millipore, Bedford, MA).

To perform the immunoassay, saliva samples were run neat or diluted 1:5 in RO water. Chlorpyrifos assays were run in antibody-coated microwell "plates" while parathion methyl assays were conducted using pre-coated "tubes". Standards at suitable concentrations were prepared in water or control (unfortified) saliva for measuring cross-reactivity (water matrix) or precision, accuracy, and reproducibility (saliva matrix). Samples and standards were pipetted into the coated wells or tubes followed immediately by the appropriate HRP conjugate. After a suitable incubation period (60 min or less), the contents were rinsed away using cool tapwater and TMB substrate was added. The resulting blue color was terminated by the addition of 1N HCl and the absorbance recorded using an EnviroQuant® tube reader or VMax® ELISA plate reader (Molecular Devices, Palo Alto, CA).

Cross-reactivity was defined in terms of "% cross-reactivity" (%X-R), which was determined by dividing the 50% Bo concentration of each reactant into the 50% Bo concentration of chlorpyrifos or parathion methyl, then multiplying by 100%. Percent Bo (%Bo) is defined to be the amount of substrate color produced in a "tube" or microwell strip by a standard or sample relative to the color produced in the negative control and expressed as a percentage.

RESULTS AND DISCUSSION

Nigg & Wade (1992) have proposed the use of saliva as a matrix for the detection, monitoring and measurement of pesticides and other chemical residues. Although saliva samples have been used for the determination of pesticides using conventional glc methods (Nigg, Stamper & Mallory, 1993), chromatographic analysis will never be used routinely for this application due to the necessity for involved sample clean-up, extraction and concentration which in turn requires a high-cost per test and the slow turnaround of results.

In contrast, using an immunoassay approach, it was found that samples need only be diluted prior to immunoanalysis.

Matrix effects can often affect immunoassay results; these effects are usually manifested as "false positives", that is, samples giving a positive result by immunoassay that are in fact truly negative for the compound of interest. False positives in an immunoassay are the result of the sample having a lower absorbance than the negative control due to excessively high or low pH, non-specific inhibition of the conjugate binding to antibody, solvent effect on the antibody or enzyme conjugate, etc. However, by diluting the saliva samples 1:5 in RO water, matrix effects were largely obviated. The limit of detection (LOD) - in the case of chlorpyrifos - was determined to be 0.17 ppb (Table 1).

Table 1. Limit of detection for chlorpyrifos in saliva

LOD = mean %Bo Unfortified saliva - (3)(mean s.d.)
Assayed in triplicate

Sample	Dilution	A450nm	%Bo	s.d.
Water	none	1.60	nd	nd
1	1:5	1.61	101%	4.16
2	1:5	1.62	101%	3.46
3	1:5	1.59	100%	2.31
4	1:5	1.59	100%	1.73
5	1:5	1.57	98%	3.00
6	1:5	1.46	91%	2.00

Mean %Bo = 98.5% Mean s.d. = 2.78

LOD = 98.5% - (3)(2.78) = 90.2% Bo
90.2% Bo = .034 ppb X 5 = 0.17 ppb

Intra-assay *accuracy* in the chlorpyrifos (CPE) plate assay were excellent (Table 2). Accuracy was measured by fortifying six different control salivas with CPE at 1.0 ppb, then recording recovery from a standard curve. In the 6 samples, recovery ranged from 59% to 107% with an average recovery of 91%. At 1.0 ppb, the c.v.'s were all less than 25%.

Table 2. Intra-assay accuracy in the CPE plate assay

Saliva samples fortified at 1.0 ppb and assayed in triplicate

Sample	Fort. level	Recov. level	s.d.	c.v.	Dilution
1	1.00 ppb	0.59 ppb	0.05	8.6	1:5
2	1.00 ppb	0.97 ppb	0.18	18.5	1:5
3	1.00 ppb	0.71 ppb	0.02	2.8	1:5
4	1.00 ppb	1.07 ppb	0.22	20.9	1:5
5	1.00 ppb	1.06 ppb	0.26	24.6	1:5
6	1.00 ppb	1.05 ppb	0.19	17.8	1:5
Mean	1.00 ppb	0.91 ppb	0.21	15.5	1:5

Precision in the CPE plate test was measured by fortifying two separate control salivas at 1.0 ppb, and again determining recovery (data not shown). In this case, average recovery and precision were measured using 12 wells per sample. The average *recovery* was 87.5% (range 86.3% to 89.2%) and the average c.v. for all 24 wells was 7.4% (3.3% to 11.5%).

Turning to parathion methyl-fortified saliva, the limit of detection in this *tube* - based immunoassay was measured in the same way as it was determined for the CPE plate assay. The LOD was 0.10 ppb (data not shown). In one intra-assay experiment (Table 3), both accuracy and precision were determined on control salivas fortified with parathion methyl at 1.0 ppb. The recoveries (accuracy) in this case averaged 132% (125% to 140%) and precision, measured as c.v.'s, averaged 10.4% (5.7% to 17.7%).

Table 3. Intra-assay precision & accuracy in the parathion methyl tube assay

Sample	Fort. level	Recov. level	s.d.	c.v.	Dilution
1	1.00 ppb	1.30 ppb	0.016	5.7	1:5
2	1.00 ppb	1.25 ppb	0.046	17.7	1:5
3	1.00 ppb	1.40 ppb	0.022	7.7	1:5
Mean	1.00	1.32	.028	10.4	1:5

The antibodies in both assays were quite specific, showing very limited cross-reactivity to only those residues that were structurally closely related. Polyclonal antisera can often have excellent specificity, despite assumptions and some reports to the contrary.

Current data was obtained primarily via fortification and recovery experiments from presumably unexposed laboratory volunteers. Further studies will be undertaken using saliva samples taken, by permission of the parties involved and in concert with the University's Committee for Human Experiments, from commercial applicators naturally exposed during the course of their work in ornamental, greenhouse and/or orchard application situations.

The two assays described are examples of the utility and power of immunoassay applications developed for environmental and human exposure monitoring. They are rapid, simple and inexpensive to perform, in contrast to chromatographic methods. The major drawback to such technology at present is their inability to provide multi-residue results and confirmation of analyte identity.

ACKNOWLEGEMENTS

The authors wish to acknowlege and sincerely thank the contributions of CSIRO, Division of Plant Industry, Sydney and Canberra, Australia and in particular John Skerritt, Amanda Hill, Helen Beasley and Simone Guihot for many stimulating discussions about environmental immunochemical technology and applications as well as their production of the high-quality antibodies used in these assays.

REFERENCES

Deichmann, W B; Herschler, D; Holmstedt, B; Keill, G (1986) What is there, that is not poison: A study of the third defense by Paracelsus. *Archives of Toxicology* **58**, pp. 207-213.

Grob, D; Harvey, A M (1949) Observations on the Effects of Tetrapyrophosphate (TEPP) in Man and On Its Use in the Treatment of Myasthenia Gravis. *Bulletin Johns Hopkins Hospital* **84**, pp. 532-566.

Harris, H; Whittaker, M (1962) Differential Inhibition of the Serum Cholinesterase Phenotoypes by Solanine and Solanidine. *Journal of Human Genetics* **26**, pp. 73-76.

Rosenberg, N M; Queen, R M; Stamper, J H (1985) Sweat-patch Test for Monitoring Pesticide Absorption by Airblast Applicators. *Bulletin of Environmental Contamination and Toxicology* **35**, pp. 68-72.

Nigg, H N; Stamper, J H (1989) Biological Monitoring for Pesticide Dose Determination. Historical Perspectives, Current Practices, and New Approaches. In: *Biological Monitoring for Pesticide Exposure* ,RGM Wang; CA Franklin; RC Honeycutt; JC Reinert (eds), ACS Symp Series **382**, American Chemical Society, Washington, DC pp. 6-27.

Nigg, H N; Wade, S E (1992) Saliva as a Monitoring Medium for Chemicals. *Review of Environmental Contamination and Toxicology* **129**, pp. 95-119.

Nigg, H N; Stamper, J H; Mallory (1993) Quantitation of Human Exposure to Ethion Using Saliva. *Chemosphere* **26**, pp. 897-906.