

Cranfield University

Department of Analytical Science and Informatics

PhD Thesis

Academic Year 2001-2004

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Early detection and monitoring of fuel leaks

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August 2005

This thesis is submitted for the degree of Doctor of Philosophy

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ABSTRACT

A novel integrated detection system has been developed to protect the environment from hydrocarbon fuels leaking from large-scale storage facilities. Over the last decade, land and subsequent groundwater contamination from hydrocarbon fuels has been identified as the major threat to drinking water resources across Europe and the United States. The integrated system developed within this work has two main components: (1) 'Early warning' free-phase fuel leak detection sensors; (2) A novel immunoassay field test for rapid, *in situ* follow-up analysis of contaminated sites through determination of the extent of the contamination and early indication of dissolved-phase fuel migration.

(1) A prototype optical reflectometric device has been developed, in conjunction with state-of-the-art data acquisition and relay technology to provide a simple, low-cost hydrocarbons monitoring solution. In extensive field trials, this system has been evaluated as a multiple-array, sub-surface, remote leak detection and monitoring system for early detection of free-phase hydrocarbon contamination. The sensors respond to all free phase hydrocarbons and can determine plume movement through soil. Preliminary leak information from these sensors can be combined with subsequent, quantitative site analysis using the immunoassay to provide an integrated, highly sensitive and low-cost solution for long term monitoring and analysis.

(2) A novel ELISA has been developed for detection of the most controversial fuel oxygenate - methyl *tert*-butyl ether (MTBE), identified as the most major threat to groundwater (and consequently drinking water) resources above any other hydrocarbon contaminant. The ELISA is a classical competitive immunoassay involving free MTBE and a novel antigen consisting of an MTBE analogue covalently bound to BSA. The ELISA was optimised and transposed into a magnetic bead format, the detection range was 0.5-5000 ppm (IC₅₀ 5 ppm, assay time 65 minutes). Also detectable are MTBE degradation products TBA, TBF and alternative fuel oxygenates TAME, DIPE and ETBE. MTBE is highly soluble in water and so is an excellent marker for assessing the extent of site contamination.

This integrated detection system potential provides a sensitive, cost-effective solution for early detection, analysis and surveillance of petroleum fuel contamination in a range of applications.

ACKNOWLEDGMENTS

I am indebted to my supervisor, Dr. Steve Setford, for his continuous encouragement and professional and personal support throughout this work.

I would like to express my sincere gratitude to Dr John Bolbot for his innovative scientific advice, personal encouragement and for the use of his computer facilities.

The WATCH European project partners (EVK1-CT-2000-00059) for their interest, scientific suggestions and warm hospitality: Dr Thomas Track (co-ordinator, DECHEMA e.V, Frankfurt, Germany); Dr. Damiá Barceló and Monica Roselle Linares (CSIC, Barcelona, Spain); Dr. Claudine Vermont-Desroches (Diaclone SAS, Besançon, France); Jens Fall (Dresden University, Pirna, Germany); Dr. Hans-Peter Rohns and Dr. Claudia Forner (Stadwerke Düsseldorf AG, Germany); Dr. Eckhart Hitsch (Salzburg AG, Salzburg, Austria) and Dr. Bjorn Jensen.

A special thank you is addressed to my daughter, Emily, for her unwavering support, patience and understanding over the years.

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LIST OF ABBREVIATIONS AND SYMBOLS

2Ab-HRP	Secondary antibody-HRP conjugate
A	Absorbance
Ab	Antibody
Ag	Antigen
ABTS	2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic acid
ASCII	American Standard Code for Information Interchange
BB	Blocking buffer
BSA	Bovine serum albumin
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
CV	Coefficient of Variation (%)
Da. (or kDa.)	Daltons (kilo-Daltons)
dia.	diameter
DIPE	Di isopropyl ether
ELIFA	Enzyme linked immuno-flowthrough assay
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
ETBE	Ethyl tert butyl ether
EtOH	Ethanol
GC-FID	Gas Chromatography coupled with Flame Ionisation Detector
GC/MS	Gas Chromatography coupled with Mass Spectrometry
GSM	Global Systems for Mobile communications
HPLC	High Performance Liquid Chromatography
HRP	Horse radish peroxidase
<i>I</i>	Current (mA)
IC50	Inhibitory concentration 50%
Ig	Immunoglobulin
K _a	Affinity constant
LOD	Limit of Detection
LOQ	Limit of Quantification
mA	milli-Amps
Mab	Monoclonal antibody
MCL	Maximum contaminant level (US)
MeOH	Methanol
MODMO	7' Methoxy-3,7-dimethyloctanal
MTBE	Methyl <i>tert</i> butyl ether
NA	Not Applicable
NSB	Non-Specific Binding
OD	Optical density
O/N	Overnight
PAb	Primary antibody
PBS	Phosphate buffered saline
PETg	Polyethylene terephthalate copolymer
PVDF	Polyvinylidene difluoride
PVP	Polyvinyl pyrrolidone
R ²	Coefficient of Regression
RSD	Relative Standard Deviation (%)
RT	Room temperature
s-HRP	Streptavidin-HRP conjugate
SB	Superblock™ Blocking Buffer
SCA	Straight chain alcohol
SD	Standard Deviation
SMS	Short Messaging Service
TAME	Tert amyl methyl ether
TBA	Tert butyl alcohol (ether)
TBF	Tert butyl formate
TBS	Tris buffered saline
TMB	3,3',5,5' -Tetra methyl benzidine
Tris	Tris (hydroxymethyl) aminomethane

V	Voltage (volts)
VOCs	Volatile organic compounds
V/v	Volume/Volume
WB	Wash buffer
W/v	Weight/volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 OUTLINE

This thesis concerns the potential environmental damage posed by the temporary storage of oil and blended petroleum fuels, of which 3,557 million metric tonnes was produced globally (per day) in 2002 (BP, 2003). Transport is an integral feature of today's society, essential for industry, commerce and recreational activities. Global consumption of oil (including crude oil, natural gas liquids, petroleum, and substitute fuels) amounted to 3,522 million metric tonnes per day, which works out at 1.25×10^{12} metric tonnes per year (BP, 2003). At any one time, fuel is continuously being produced, stored, traded and transported across the world. Since the 1950s and the post World War 2 economy boom, ever-increasing demands for petroleum fuels necessitated multiple constructions of large, underground fuel storage tanks (USTs), underground pipelines and petrol filling stations. The majority of such contain petroleum products, such as petrol/gasoline, diesel, heating oil, kerosene and jet fuel, although many other chemical substances are also stored in USTs.

Since the late 1980s, it has become increasingly apparent that natural corrosion of USTs, along with faulty installation and operation procedures, has resulted in severe leakage problems, particularly in the US, which has caused widespread land and groundwater contamination. By 1999, the United States Environmental Protection Agency (US EPA) was monitoring ~370,000 leaking UST sites ('LUSTS') across the US and estimated that approximately 21,000 site clean-ups were necessary for the year 2001. Remediation of such sites is now funded by the US EPA's LUST Trust Fund, which was then funded at ~\$70 million per year (Ryan, 2004).

Contamination from organic pollutants has also been identified as a major risk to international water resources. Hydrocarbon leaks migrate down through the soil horizons to the groundwater table. The more soluble compounds present in petroleum fuels, such as benzene, toluene, ethylbenzene and *n*-xylenes (BTEX) and more recently, the fuel oxygenate methyl *tert* butyl ether (MTBE), are of particular concern because of the specific threat they pose to local groundwater resources and drinking water supplies.

This first part of this Chapter (1.2-1.3) gives an overview of the environmental problems presented by leaking USTs and of the ever-increasing problems associated with MTBE, with a review of current analytical methods of detection for these compounds. In the light of this

information, it is clear that there is a need for rapid and sensitive field-based sensors that can be employed for remote monitoring of USTs in order to provide early detection of hydrocarbon fuel leaks. In addition, there is a clear need for a field-based analytical test for the detection of MTBE. When combined together, these two analytical tools can provide an integrated ‘early-warning’ system, which can alert site operators to the incidence of a leaking UST and then be used to determine the extent of the problem and monitor the movement of the petroleum fuel/MTBE contaminant plume.

1.1.1 Aims & objectives of this work

The specific aims of this work are to provide an integrated two-part system for the early detection and preliminary assessment of leaking USTs. The objectives can therefore be defined as:

- To develop and evaluate a continuous, remote sub-surface sensor-based monitoring system capable of monitoring USTs and tracking leaked hydrocarbon plumes.
- To develop and evaluate a novel, rapid and sensitive immunoassay field test for the fuel oxygenate MTBE, which can be used subsequent to the early leak warning given by the sub-surface sensor system in order to provide an early indication of the extent and direction of the plume movement.

The second part of this Chapter (1.4-1.6) gives an introduction to the experimental approaches taken within this work to accomplish this.

1.2 UNDERGROUND STORAGE TANKS (USTs)

The US EPA regulations define an underground storage tank (UST) as ‘a tank and any underground piping connected to the tank that has at least 10 percent of the combined volume of the tank underground’. US EPA regulations apply only to underground tanks and piping storing either petroleum or certain hazardous substances. Up until the late 1980s, USTs were generally constructed of bare steel. The average life expectancy of a steel tank was 30-50 years. Under a typical petrol station, there will be 4-5 large (10,000 gallon) underground tanks, one for each fuel type (unleaded, premium unleaded, diesel, LPG) and one for waste fuel (forecourt run-off). These USTs are regularly filled by tankers, with fuel products delivered to the pumps by an underground pipe system. Such USTs and pipelines all have the potential for leakage and spills during refilling are common.

To put a UST fuel leak into perspective, a slow 10 gallon (in total) petrol leak from a 10,000 gallon UST at a petrol filling station could be virtually undetectable to the station operator using manual gauging and inventory controls, but poses a significant threat to groundwater. Assuming the benzene content of typical petrol is 0.76% (v/v), a 10 gallon leak (0.1% of the total inventory) contains 230 g benzene (SG 0.805 g/ml). With a drinking water standard of 5 ppb (as in the US), 10 gallons of leaked petrol therefore has the potential to contaminate (and fail drinking water quality standards) 12 million gallons (46 million litres) of groundwater (Ryan, 2004). Operation of fuel storage and delivery is the overall responsibility of the fuel station owner.

In the US, the EPA estimates that since the federal underground storage tank (UST) program began, nearly 1.2 million of the roughly 2.1 million petroleum tanks subject to regulation have been removed from service, leaving approximately 919,000 active USTs. As of June 1998, more than 358,000 releases had been identified, nearly 302,000 clean-ups had been initiated, and 192,000 clean-ups had been completed (Squillace *et al.*, 1999). The US EPA expects that many more leaking USTs will be identified as owners and operators act to comply with leak prevention and detection regulations that took effect in December 1998. Discovery of more releases seems likely, given that the EPA estimated in October 1998 that roughly 40% of the 919,000 active tanks had yet to be upgraded, replaced, or closed.



Fig. 1.1 Excavation of corroded & leaking UST

Corroded USTs (shown left) and decommissioning/excavation of a petrol forecourt UST (right). Source: Ryan, 2004.

As a result of increasing information and concerns about LUSTS, the US responded with new legislation which required owners of existing USTs (tanks installed before December 1988 and over 110 gallon capacity) to upgrade, replace, or close their USTs by December 1998, which required expensive, large scale excavations (Fig. 1.1).

The revised US EPA regulations now require new storage tanks (post 1998), whether above (Fig. 1.2) or below ground to meet minimum standards and include the following leak protection requirements:

- Corrosion protection for tanks and piping: tank and piping must be constructed of non-corrosive material (generally steel/epoxy coated or fibreglass materials)
- Spill protection: catchment basins, proper tank filling practices
- Overfill protection: automatic shutoff devices, overfill alarms, or ball float valves
- Secondary containment; a tank liner, and/or surrounding vaults or concrete bunds
- Automatic tank gauging systems: monitoring of product level and temperature
- Manual tank gauging: dipstick monitoring of tank contents (for tanks <2,000 gallons)
- Groundwater monitoring: monitoring wells for detecting liquid product floating on the groundwater table
- Vapour monitoring: monitoring wells for detecting product vapour in the soil around the tank and piping
- Tank tightness (pressure) testing
- Statistical inventory control: statistical analysis of inventory, delivery, and dispensing data

The UK has closely monitored the situation in the US and has adopted a similar legislative approach. Oil is the most common pollutant of water resources in the UK and the Environment Agency (EA) currently responds to $\geq 5,000$ reported incidents per year, many of which could have been avoided or the environmental impact reduced by better storage (McNamara, EA, 2004). An EA evaluation (in 2001) was that 60% of UK oil storage tanks were either unbunded or inadequately banded and, as a result, were five times more likely to be involved in a pollution incident.

In response to this information (and acknowledging the fundamental changes in the US), 'The Control of Pollution (Oil Storage)(England) Regulations 2001' were introduced. These require all existing facilities storing more than 200 litres of oil (all types) to have a secondary containment system, such as a bund (a brick/mortar or concrete sump placed underneath a tank to contain leakage), by 01 September 2005. Bunds must be able to contain 110% of the tank capacity. All new tanks have to comply with the new regulations and the EA will only accept integrally banded steel tanks, not to be confused with double-skinned tanks, which, under the new legislation, are still only classified as 'enhanced primary containment'. To clearly define the revised legislative requirements and assist ground fuel/oil storage, 'Pollution Prevention

Guidance notes (PPGs)' were revised and reissued (PPG3s 2001) by all the UK EAs (England & Wales, Scottish EPA) and the Irish Environment & Heritage Service.



Fig. 1.2 Modern (post-2004) above-ground fuel storage tanks

Commercial 'Luton AG' petroleum storage tanks (left) and a 'Teeside' storage tank (right). The new style above-ground storage tanks are sited above the secondary concrete containment bund.

A host of additional UK regulations apply for the content and storage of all fuels (leaded, unleaded, super 'high octane' unleaded and diesel) which have to meet a number of British Standard (BS) requirements that also embrace EU Directives. An example, but by no means exhaustive, list of primary UK legislation is given, as follows:

- The composition and content of fuel used in motor vehicles is regulated in the UK under The Motor Fuel (Content & Composition) Regulations 1994 (SI2295), which was initiated under the Clean Air Act 1993.
- Petrol storage and loading from road tankers is regulated by licence under the Petroleum (Consolidation) Act of 1928 (PCA), by Harbour, Fire and Civil Defence Authorities and County and Unitary Councils (EA, 2001).
- For oil storage at sites other than refineries or distribution depots, tanks, pipework and structures are all required to comply with BS or OFTEC (Oil Firing Technical Association for the Petroleum Industry) standards and must be tested under a quality assurance system complying to ISO9001/2.
- The DETR Oil Storage Regulations require minimum standards (BS) for industrial and commercial storage facilities, which encompass tank designs and containment systems (EA, 2000, 2001).
- The Health & Safety Executive (HSE) have additional policy requirements, in conjunction with a number of detailed, non-mandatory guidelines issued by the Environment Agency.
- Filling stations storing & dispensing fuel must comply with the PCA 1928 and the Health & Safety Guidance Document for underground storage tanks HS(G)41 (EA, 2001).

- Above-ground storage tanks must meet the requirements of the HS(G)50 and HS(G)51 guidelines, in conjunction with Environmental Agency PPG2 guidelines (EA, 2001).

In general, these provisions are considered a major step forward in preventing leaks and subsequent groundwater pollution. However, these requirements are still not considered acceptable by some environmental organisations reviewing the regulations. Indeed, whilst storage tank regulations have improved, any UST can still leak, quite probably for the same reasons as the steel USTs, such as installation damage, faulty connecting valves or poor operating procedures. Natural corrosion or deterioration of many modern replacement materials and/or their resistance to underground stresses are not yet known. Above-ground storage tanks rely on the integrity of their masonry/concrete containment bunds, but these materials are also prone to natural degradation. Such systems still rely upon regular, manual inspections. Therefore, there is clearly a requirement for automated, UST leak detection/monitoring systems in remote sites.

1.3 PRIMARY ORGANIC CONTAMINANTS OF CONCERN

1.3.1 Petroleum fuels (petrol)

‘Petrol’ is a complex mixture of many hydrocarbon constituents. Between 15 and 500 compounds may be blended together to create different fuel blends. Petroleum composition consists of saturated or unsaturated hydrocarbons having 3-12 carbons. A typical formulation contains 62% alkanes (such as *n*-butane, *n*-pentane, isobutane), 6% alkenes and 31% aromatics (including BTEX). However, up to 18% of alcohols, ethers (including MTBE) and other additives can be included in this blend (Nihlén, 1998). Fuel oxygenates, such as MTBE, can be blended in volumes of between 5-15% to improve the octane rating, which increases fuel burning efficiency (EC, 1998, Moran *et al.*, 1999). Under UK DETR guidance classification, ‘petrol’ represents all leaded, unleaded and liquid petroleum gas (LPG), but excludes diesel, kerosene and aviation fuels (Morley, 1999).

1.3.2 BTEX compounds

Because most LUST sites are contaminated by petroleum fuels, concerns have traditionally concentrated on non-aqueous phase liquids (NAPLs) and the toxicological fractions from mineral oil-related compounds. Specifically, these were the dissolved phase BTEX aromatics (benzene, toluene, ethylbenzene and *m*, *o*, *p* – xylenes, Fig. 1.3). These BTEX compounds are grouped together due to the similarity of their organic structures and are commonly used as an indicator for petroleum contamination. BTEX compounds generally account for 18% (w/w) of

standard petroleum blends. Benzene, which can be present in fuels between 1-11% is known to have significant human carcinogenic potential. It is also the most soluble (1880 ppm at 25.5 °C) BTEX compound. Long-term exposures to benzene in drinking water at levels above 5 ppb (the US EPA's Maximum Contaminant Level - MCL) have been shown to increase the risk of cancer. Toluene (MCL-1 ppm), ethylbenzene (0.7 ppm) and *n*-xylenes (10 ppm) are not carcinogenic but can cause damage to the liver, kidneys, and the central nervous system (CNS) after long-term exposure (US EPA, 1994/1997).

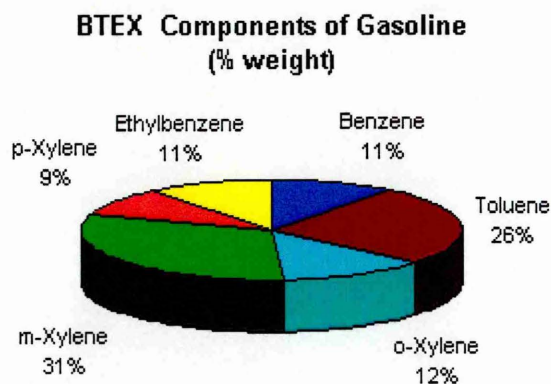


Fig. 1.3 Typical BTEX components present in petrol

The pie chart shows the average percentage weights of total BTEX components in normal gasoline (petroleum) fuel (usually ~ 18% w/w in petroleum blends).

1.3.3 Fuel Oxygenates

From the early 1970s, increasing concern about public health and environmental impacts from exhaust emissions led to changes in the formulations of motor fuels. Revised European and US regulations were targeted to reduce emissions from motor vehicles and improve air quality. Fuel additives are generally derived from alcohols, ethers and metals, and are classed as either anti-oxidants, anti-icing agents, anti-knock agents or octane enhancers (oxygenates).

Oxygenates are oxygen-containing compounds, such as alcohols or ethers. An ether is typically a compound containing an oxygen atom bonded to two carbon atoms. Petrol or 'gasoline' fuels burn cleaner when blended with oxygenates, as these blends are designed to compensate for varying oxygen levels within the engine system. As a result, fuel combustion is more efficient and generally results in less toxic exhaust emissions to the atmospheric environment. Oxygenated compounds were subsequently incorporated in fuels to aid combustion, reduce exhaust emissions (responsible for air pollution) and to aid fuel producers to reach the octane levels required by new fuel standards (EA, 2001). The choice of oxygenate (Fig. 1.4) varies between countries and US states, primarily influenced by the availability of raw materials, individual economies and political status. Oxygenates also act as anti-knock agents, preventing

fuel from igniting prematurely and causing engine damage and are now preferred to other metal-based anti-knock agents (such as triethyl lead or methylcyclopentadienyl manganese tricarbonyl) due to the high costs and incidences of environmental contamination from these compounds. The fuel oxygenates MTBE and ETBE have been used extensively to achieve mandatory fuel standards throughout the US and Europe, as well as achieve air quality standards (1.1.3.4).

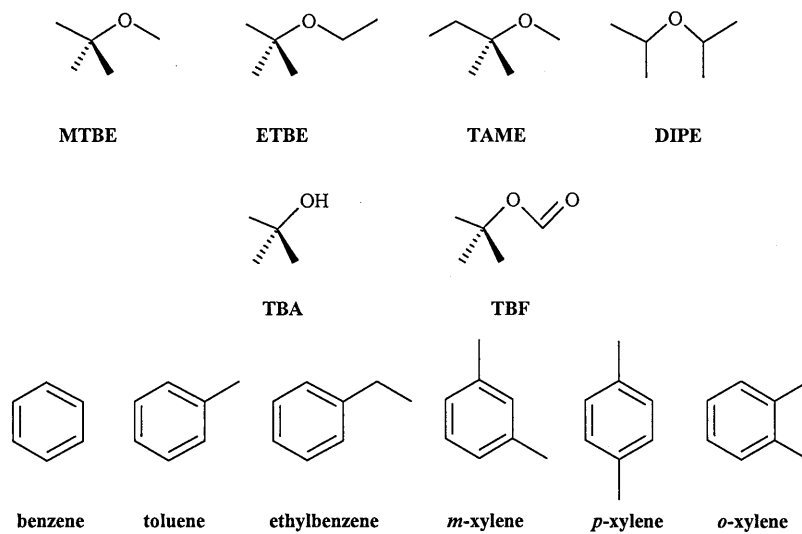


Fig. 1.4 Chemical structures of common fuel oxygenates and BTEX aromatics

All BTEX compounds are a single benzene ring with one or two methyl groups (CH_3) attached. The fuel oxygenates methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE), *Tert*-amyl methyl ether (TAME) and di-isopropyl ether (DIPE) are all branched ethers with methyl groups. *Tert*-butyl alcohol (TBA) and *tert*-butyl formate (TBF) may also be blended in fuels (these two compounds are the also the natural breakdown products of MTBE).

1.3.4 MTBE

MTBE (Fig. 1.4) was first blended into petroleum to increase the oxygen content of petrol in order to improve air quality under the 1990 US EPA Clean Air Act. MTBE was the preferred oxygenate due to its high octane value, low cost, ease of production and favourable transfer and blending characteristics (Squillace *et al.*, 1995). Research has shown that MTBE blended fuels decrease emissions of CO, aromatics, 1-3 butadiene and ozone and reduce overall the odour detection threshold of exhaust fumes (Nihlén, 1998). MTBE is highly soluble in water (Table 1.1) and is also miscible in other ethers, alcohols and petroleum fuels, blending without phase separation (EC, 1998). The key properties of MTBE are shown in Table 1.1.

At room temperature (24 °C), pure MTBE is a volatile, flammable, colourless liquid with a terpene-like odour (US EPA, 2005). MTBE is manufactured from a mixture of methanol (~ 36%

by mass) and isobutylene, both produced by oil refineries (EC, 1998). The methanol used is usually derived from natural gas or coal, but may alternatively be produced from ‘biomass’ (a non-fossil fuel) and so can be perceived as a ‘sustainable’ energy source. Within a refinery stream, methanol and isobutylene are reacted over a catalyst at moderate temperature and pressure to yield a product containing >80% MTBE. This is then dried and purified to a purity of >95% MTBE. MTBE can also be manufactured through the dehydration of *tert*-butyl alcohol (Nihlén, 1998).

Table 1.1 Physical & Chemical Characteristics of MTBE & ETBE

The table shows MTBE characteristics compared to ETBE, the second most popular fuel oxygenate (Goering, 2000; Nihlén, 1998; US EPA, 1994).

	MTBE	ETBE
Synonyms	Methyl tertiary-butyl ether, MTBE, methyl <i>t</i> -butyl ether, 2-metoxy-2-methylpropane, methyl <i>tert</i> -butyl oxide, <i>tert</i> -butyl methyl ether, methyl 1-1,1-dimethylethyl ether, 1,1-dimethylethyl ether	Ethyl tertiary-butyl ether, ETBE, 2-etoxy-2-methylpropane, ethyl <i>tert</i> -butyl oxide, <i>tert</i> -butyl ethyl ether, ethyl 1,1-dimethylethyl ether, 1,1-dimethylethyl ethyl ether
Molecular formula	C ₅ H ₁₂ O	C ₅ H ₁₄ O
Molecular weight g/mol	88.15	102.18
Boiling point °C	55.2	73.0
Specific density g/cm ³	0.7404 (25°C)	0.7364 (25°C)
Water solubility g/100g H ₂ O	4.8	1.2
³ Water solubility g/litre	23.2-54.4	
² Odour detection threshold	53 ppb	13 ppb
⁵ Taste Threshold in water	134 ppb	47 ppb
Ignition temperature °C	224	N/A
⁶ Reid vapour pressure (RVP)	N/A	4.0
Vapour pressure kPa (25°C)	32.7-33.5	17.3

N/A – data not found

¹ Obtained by calculating the molecular mass of each component

² These figures vary by individual countries, American states and authors

³ (Squillace *et al.*, 1995)

⁴ Conversion factors (20°C, 101.3 kPa)

1 ppm = 3.605 mg/m³ 1 ppm = 4.24 mg/m³
1 mg/m³ = 0.278 ppm 1 mg/m³ = 0.236 ppm

⁵ Nihlén (1998)

⁶ Reid vapour pressure is a measure of volatility. For gasoline/petrol, the lower the RVP, the lower the exhaust emissions. RVP of gasoline/petrol can vary between 7-9 psi.

By 2002, MTBE (up to 15% v/v) was used in 80% of all oxygenated fuels (leaded, unleaded and premium grade fuels). UK refiners currently add >10% MTBE by volume in leaded and unleaded fuels. For conventional economy fuels, basic blend petrol is simply mixed with MTBE-blended fuels at the refineries and the resulting blends contain lower concentrations of MTBE, generally between 1-8% (Squillace *et al.*, 1995). In super or premium grade unleaded fuel, MTBE is added in its pure form at higher concentrations, legally >15% v/v (EA, 2000).

1.3.4.1 Environmental problems with MTBE

Over the last decade, MTBE has become one of the most significant environmental pollutants, particularly in groundwater and drinking water reserves. Poor management of stored fuel and UST leaks have resulted in major, global environmental contamination from petroleum and related components. The US Geological Survey's (USGS) National Air & Water Quality Assessment (NAWQA) Programme during 1990-1998 found high incidences of MTBE contamination in both confined and unconfined ground water aquifers and drinking water (Lince *et al.*, 2001). The survey reported that 20% of groundwater in areas where reformulated gasoline was sold was contaminated by MTBE, with as many as 9,000 community water wells in 31 states potentially contaminated (Moran *et al.*, 1999/2000). The main source of this oxygenate contamination has been shown to be oxygenate-blended petrol, from a combination of leaking USTs, petroleum facilities, transfer spillage, and accidental spills (EA, 2001, US EPA, 2005). Due to MTBE's high solubility in water (51.26 g/l), surface water systems may become contaminated with MTBE from ground waters, atmospheric deposition, storm water run-off and direct releases by industrial and recreational activities (Bradley *et al.*, 1999).

In March 1999, California announced an Executive order (D-5-99) to phase-out MTBE from gasoline fuels by 2002, following heavy losses of groundwater supplies through MTBE contamination (EA, 2000). This was extended for a further year (to 2003) to enable refineries to make the transition to ethanol. Ethanol was already a popular alternative to MTBE, marketed and distributed in every US state as a 'clean' octane source. In 2001, 60% of MTBE demand (29.4 million metric tonnes) was from North US states, whereas ethanol was more popular in the East and Midwest (RFA, 2005). California has been the pioneering state in phasing out MTBE and the effects on the economy, energy demand and its trade patterns in the US, Europe and Asia has been closely studied. In a 2001 public statement, California announced that there had been a 600 million gallon increase in ethanol demand, extensive expansion of new ethanol plants, manufacturing ethanol from agricultural waste and indigenous feedstock, which in turn had benefited rural and state economic benefits. The 'sustainable' aspect of using ethanol (a renewable resource) has been greatly emphasised, along with its environmental benefits. Ethanol is considered non-persistent, biodegradable and poses no health threats (RFA, 2005).

In response, the US Department of Energy have since placed a 3% volume cap on MTBE in fuel and the US EPA have announced that they intend to remove ether oxygenates from *all* petroleum fuels throughout the US (Goering, 2000, RFA, 2005). According to recent reports, expanding the ethanol industry to meet the additional demand created by an MTBE phase out would result in \$1.9 billion in new investment in both expansions to existing production

facilities and the development of new plants in the U.S. Most of the MTBE produced in the US is from US refineries that can easily switch from producing MTBE to other products, resulting in negligible employment losses. Increased development and investment in such plants will also remove the need for MTBE imports from OPEC countries (RFA, 2005).

In Europe and the UK, the situation regarding potential MTBE contamination is less clear. The threat to ground waters by leaking USTs has been recognised for the past 20 years, but through differences in political and environmental agendas, there is no single approach to UST management in Europe (EA, 2000). A 1996 UK survey showed that 5 out of 8 EA regions had recorded incidences of MTBE in groundwater. However, in 1999, only one UK water company routinely monitored for MTBE, and no special attention is paid to monitor water supplies located close to petroleum outlets, where the risk of leaking USTs and spillage is highest (EA, 2000). Mineral oil-related compounds, mainly hydrocarbon fuels, are responsible for 90% of all hazardous organic contamination across Europe. This represents approximately 50,000 sites, many located near the incoming flow of water plants. These contaminated sites are now considered the primary risks to European water resources as groundwater reservoirs supply 60-70% of all drinking water, with more than 3,000 public water supply and industrial water supply boreholes, along with many, smaller, private sources in rural areas (NGCLC, 2000; Bro-Rasmussen, 2000). As a result, there are increasing European demands for the phasing out of MTBE.

1.3.4.2 Effects of MTBE on human health

Currently, MTBE is still tentatively classified in the US as a potential human carcinogen, based on certain animal toxicity studies (US EPA, 1994, Research Triangle Institute, 1994). However, this is still widely debated as carcinogenic effects (in mice) only occurred after extremely high exposure concentrations (>7973 ppm) over an 18 month period (Squillace *et al.*, 1995). Later studies on the potential toxicity of MTBE has resulted in the general opinion that MTBE (and TBA) is very low in toxicity (US EPA, 1997; NHSE, 2000a; Nihlén, 1998).

Whilst MTBE is rapidly absorbed by both respiratory and gastrointestinal tracts and blood levels of MTBE increase rapidly on exposure, levels decrease soon afterwards (2-4 hours) primarily through exhalation and urinary excretion (Nihlén, 1998). MTBE (and other ethers) is metabolised by oxidative demethylation and *in vitro* experiments have shown that MTBE is metabolised to the primary metabolites tertiary butyl alcohol (TBA) and formaldehyde, with possible formation of methanol, formic acid, 2-methyl-1,2-propanediol, 1-hydroxyisobutyric

acid and carbon dioxide. TBA can also be biotransformed to formaldehyde. (Research Triangle Institute, 1994; Squillace *et al.*, 1995).

The Research Triangle Institute (1994) stated that MTBE and its metabolites have not been found to bioaccumulate in tissues in humans or surface-water aquatic organisms. Later evidence suggested that it can be deposited in body stores and excreted over a longer period of time. It has also been detected in breast milk (Squillace *et al.*, 1995; Nihlén, 1998). Nihlén (1998) studied the respiratory effects of MTBE and ETBE on human subjects and reported very small or negligible effects from MTBE at levels >50ppm. In contrast, elevated irritations of throat and airways in human subjects were found after ETBE exposures (25-50 ppm), with minor pulmonary lung function impairments in excess of 50 ppm.

The UK's 'Committee on the Medical Effects of Air Pollutants' (COMEAP) statement of September 2000 concluded that 'there were no significant health risks associated with inhalation and exposure of MTBE added to petrol at low ambient air concentrations in the UK' (NHSE, 2000a). Ambient concentrations whilst refuelling at petrol stations were estimated to be ~10 µg/m³ and no greater than 30 µg/m³ (NHSE, 2000a). The 1996 Health Effects Institute noted that employees involved in handling of MTBE (refineries for example) may be exposed to atmospheric levels >1000 ppm (NHSE, 2000a). Neurotoxic effects (in rats) have been shown to impede motor activity at 800 ppm (3142 mg/m³) with sedation and ataxia at higher concentrations (NHSE, 2000a). However, in a later COMEAP meeting (06/10/00), it was argued that more information on exhaust emissions and the fate of MTBE was necessary, particularly with regard to formaldehyde, which is formed during the combustion process and also during metabolic activity, as this has been implicated as a cause of occupational asthma (NHSE, 2000b). US concerns regarding MTBE affecting air quality, health, and water quality are excellently reviewed in a 2001 US Congress Report (McCarthy & Tiemann, 2001).

1.3.4.3 Drinking water limits for MTBE contamination

Whilst the potential toxicity of MTBE is still debated, it has a very low taste and smell threshold (between 5-45 ppb) which has been the primary cause of drinking water supply losses. The US still has no fixed MTBE maximum contaminant level (MCL) for drinking water, although between 20-40 ppb has been advised, but this varies by state (US EPA, 1997). Internationally, countries are also divided over the MTBE issue and contamination limits in drinking water can vary between 5-50 ppb. Regardless of what 'safe' levels are set and by whom, what is generally accepted is that at ~20 ppb, MTBE can render drinking water unfit for human consumption because of its unpleasant taste and odour.

1.3.4.4 Contaminant Phase Movements through soils

All surface petroleum contaminants will migrate through the subsurface (Fig. 1.5). They will be found as free-phase products, adsorbed or bound to soil particles, or in interstitial spaces in the geological matrix, or dissolved into the groundwater (LaGrega *et al.*, 1994). Contamination, concentration and distribution will be strongly influenced by site-specific factors, such as soil characteristics (permeability, type and structure), distance from the point source of pollution, geological bedrock formations, oxygen availability, built constructions, buried wastes, tree roots and microbiological activity. The fate and transport mechanisms of contaminants are also affected by each contaminant's chemical characteristics, which may widely vary. In general, the processes of volatilisation, dissolution, sorption, solubility and degradation determines the phase transportation through soils and the concentrations of soluble compounds which arrive in groundwater systems

MTBE is much more water-soluble (51.26 g/l) than benzene (1.8 g/l) and so is readily dissolved into groundwater. The remaining BTEX compounds (Fig. 1.3) are less soluble (especially ethylbenzene & *n*-xylenes <0.5 g/l) and more readily sorbed to aquifer sediments ('sorption' is either the attachment of the organics to the soil surface or the intake of the organics into the soil matrix). Various studies of benzene contamination of groundwater by leaking USTs in Texas have reported that most benzene plumes extend <80 metres from their source (Mace *et al.*, 1997). MTBE, through co-solvent effects can increase the solubility of BTEX (Ray *et al.*, 2003). It is generally accepted that MTBE plumes extend much further than the general contaminant plume without significant degradation (half-life of 10,000 days, Ray *et al.*, 2003). In some cases, MTBE transport has exceeded the transport distances of BTEX compounds ten-fold (Newall *et al.*, 1998). As a relatively 'new' contaminant, MTBE plumes have proved difficult to detect and delineate using traditional analytical methods (Nichols *et al.*, 2000).

1.3.4.5 Analytical methods of detection for fuel contamination

The environmental sector has seen explosive growth in innovative technologies for contaminated site assessments as a result of greater awareness of leaking USTs. Conventional methods for tracing leaked fuel plumes and collecting soil and water samples require extensive sub-surface drilling and the construction of ground water monitoring wells, usually in conjunction with extensive geophysical surveys and flow modelling tools.

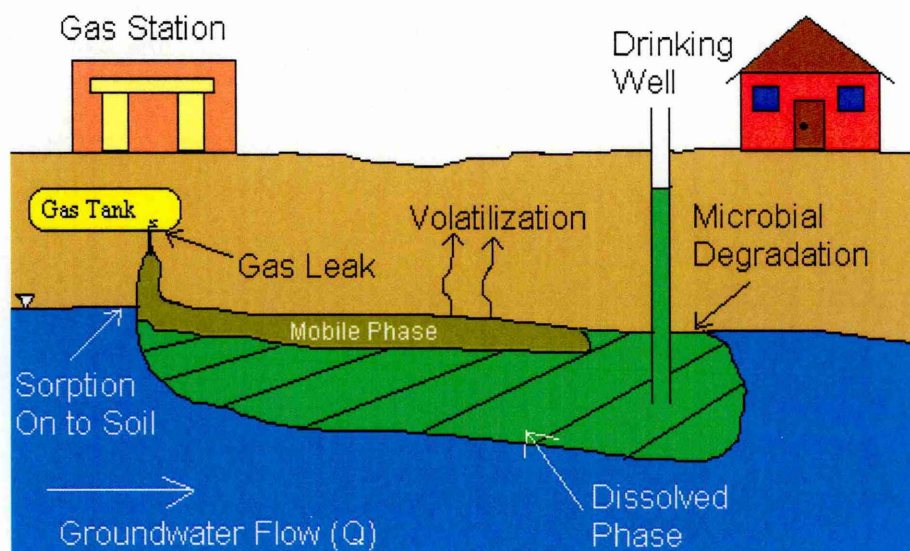


Fig. 1.5 General schematic of sub-surface contaminant pathways

Contaminant plumes form within the soil matrix from leaking USTs, with heavier fractions adsorbing onto soil particles and becoming trapped within interstitial spaces (pores). The more soluble components, such as benzene and MTBE dissolve into soil water and migrate towards groundwater, which in turn carries the contaminants towards drinking water extraction wells.

Classical laboratory-based analytical methods for detection of hydrocarbon fuels and associated fuel oxygenates are the US EPA Method #8260 ‘Volatile organic compounds (VOCs) by Gas Chromatography/Mass Spectroscopy’ (GC/MS), Methods #8020/8021 (using GC with Photo Ionisation Detectors, PID) for testing fuel oxygenates (MTBE, ETBE, DIPE and TAME) and Method #8015 ‘Aromatic total petroleum hydrocarbons (TPHs) by Gas Chromatography/Flame Ionisation Detector’ (US EPA, 2003; Nichols *et al.*, 2000). For all of these techniques, analytes are stripped from the sample using purge and trap (P&T) and separated by GC. Whilst Methods #8020/8021 are relatively inexpensive techniques, the presence of TPH can interfere with the PID and prevent accurate oxygenate measurements (Happel *et al.*, 1998). GC/FID is less sensitive than the other methods (measuring in the ppm range only) and a major disadvantage with FID is that the technique is destructive (VOCs are combusted during the ionisation step) and so samples are no longer suitable for further analysis (RAE systems, 2002). Only Method #8260 (GC/MS) can identify and distinguish oxygenates accurately when other fuel hydrocarbons are present, but a major drawback is that this is an expensive and complex analytical method (Nichols *et al.*, 2000). Conventional site characterisation tools and well installation procedures are described in API (1996a) and more innovative characterisation and monitoring technologies are thoroughly reviewed in US EPA (1997b).

On-site sampling tools and mobile labs are now also used, but these still depend on conventional and relatively expensive analytical methods, such as portable GC and GC/MS (Matz *et al.*, 1997). However, such on-site sampling methods may not be suitable for all release site assessments, such as sites overlying bedrock, where conventional drilling and monitoring wells are still necessary, and so samples must still be manually collected and forwarded to conventional laboratories for analysis.

The accuracy of off-site analysis is still debated, primarily due to the potential for volatile losses during the sampling processes and inefficient sample collection and preservation/transportation methods. Using traditional laboratory facilities requires that environmental samples first be taken in the field, transported to the lab, analysed within the laboratory work schedules and the results report then prepared and relayed back to the relevant person. Inherent in such centralised testing regimes are three major disadvantages.

1) *Excessive sample turnaround time* (time from taking the sample to receiving the result). Clearly this delays appropriate action at the point of sampling, and in some cases may compromise the quality of monitoring if retrospective action is delayed.

2) *Excessive sample size*. In the absence of a field test, many more field samples need to be sent to the lab merely to *screen* the site for contaminated areas. An effective, low-cost field test allows for efficient on-site screening in which an operator may be able to predict and then track contaminant plumes. Centralised tests may still be used to some extent for final confirmation/validation, but the much smaller sample size, which would be required for this will clearly have an impact on cost.

3) *Sample stability*. The delay time inherent in the centralised laboratory testing method, between taking the sample and its eventual analysis, can extend to several days, or even weeks, sometimes under unpredictable conditions. This can lead to critical changes in sample content due to precipitation, chemical breakdown or microbial decomposition, resulting in highly inaccurate monitoring data.

For these reasons there is great interest in a range of rapid and reliable field tests for both leak detection and for on-site analysis of leaked petroleum contaminants. Both of these have been addressed within this work (Section 1.4).

1.4 INTRODUCTION TO THE EXPERIMENTAL WORK

1.4.1 Emerging fuel leak detection technologies

Traditional detection methods of leaking USTs generally relied upon manual dipstick testing levels within tanks and comparing this to fuel inventories. Once a leak or spill had been ascertained, the extent of the leak had to be ascertained through soil sampling, drilling monitoring wells and repeated water sampling. These samples have then to be forwarded to laboratories for analysis using more traditional methods (1.3.4.5). However, in response to the revised legislation regarding the storage of petroleum fuels (and other hydrocarbons), over the past few years, a number of competing technologies for detection of hydrocarbon leaks from storage facilities have emerged, indicating the widespread acknowledgement of the importance of this problem. Some of these most popular technologies are described, as follows:

Monitoring wells: The more traditional leak detection method is the siting of monitoring wells (boreholes) in suitable locations around USTs, with regular, manual water sampling and subsequent sample analysis required. More modern adaptations now include sensors for detecting hydrocarbons. These may be either buried in surrounding soil, or placed in monitoring boreholes around the tank. Such probes may measure vapour (volatile organic compounds, VOCs) or detect oil sheens on groundwater surfaces within the boreholes, such as the Leakwise® ID series Oil Sheen on Water monitoring systems (Ionic Instruments, Boulder, USA). These floating sensors use high frequency electromagnetic energy absorption technology that enables monitoring of hydrocarbons on water surfaces. A high frequency transmitter is connected to the floating 'antenna', the amount of energy absorbed by the surrounding fluid matrix is then measured (the principle being that water absorbs more energy than hydrocarbons).

Automatic Tank Gauging (ATG): This technique uses a measuring probe, such as a floating probe, situated within the tank, which measures the level of fuel. Computer-based reconciliation between the fuel level and the amount of fuel dispensed can be used to give a warning of a potential fuel leak, but this is not detection of fuel leakage per se. This method is more useful as an inventory tool and so is relatively insensitive. Small fuel losses over a long period of time may not be detected if there is not a significant drop in tank level, especially as a typical UST capacity is 10,000 gallons. ATG equipment is supplied by a variety of manufacturers, such as Veeder-Root (Richmond, UK), Incon® and EBW® (Franklin Fuelling Systems Inc., Madison, USA), amongst many others and the technologies used may widely vary.

Interstitial Detection: A variety of methods are in use for detection of leaked fuel into the interstitial space between the two walls of a double-skinned tank. The most simple of these uses a liquid barrier between the walls, such as the TSP-HIS Interstitial Hydrostatic Sensor (EBW®, Franklin Fuelling Systems), which is supplied with a header tank with a level measurement. The interstitial space is filled with brine and, as fuel leaks into the interstitial space, the level of the brine in the header tank increases. Alternatively, the increase in pressure can be measured. Another method is to induce a vacuum between the walls of the tank and to then monitor the level of the vacuum – a drop in vacuum indicates a leak.

More elegant interstitial sensors are now available, such as the TSP-DIS (EBW®, Franklin Fuelling Systems), which are electro-optical sensors that can detect hydrocarbons and/or water present in the interstitial space, in a similar method to the sub-surface sensors developed within this work. Other probes can be electroconductive switches, which, in the presence of hydrocarbons, produce an electrical signal. Alternative sensors may also be cables that can then detect leaks in either the interstitial space or surrounding pipework, for example LiquidWatch® (Permapipe, Illinois, USA), which can detect water or hydrocarbon leaks in secondary containment systems and/or sumps.

1.4.2 Development of a sensor-based sub-surface fuel leak monitoring system

There is still clearly a market for a reliable, continuous, sensitive and automated fuel-leak detection system, which can be placed sub-surface around new and existing USTs, where practicable. Of key importance is that the sub-surface sensors developed within this work should be able to discriminate between hydrocarbons and water. Another key consideration is that they should be designed to be highly adaptable and so can be employed at many sites, such as directly buried in soil around storage sites, placed within interstitial tank spaces and/or secondary containment systems and possibly monitoring wells. The design and chronological development of these sub-surface sensors, together with an integrated, continuous data logging and data relay system for underground fuel leak monitoring is extensively presented in Chapter 2 of this work, with the developmental methodologies and results provided in Chapters 3 & 4 respectively.

However, another equally important aspect of this work is that the sensors should be used as part of an integrated system, which can provide an early warning of a leak event, followed by on-site characterisation tools to clearly evaluate the extent of the leak and monitor the

movement of any leaked contaminant. This can be achieved by incorporating the sensors with sensitive and rapid immuno-based analytical tools.

1.4.3 Integration of sub-surface sensors with immunodiagnostic tools

Whilst the sub-surface sensors will respond to general hydrocarbon populations in the soil, in order to provide a more detailed initial field-based characterisation of an early contamination problem, any triggering of sub-surface sensors could be followed by the use of immunoassay-based methods for the specific characterisation of key target analytes within the vicinity of the triggered device(s). For example, in a petrol station forecourt, there may be 4-5 USTs containing different hydrocarbon fuels. Rapid characterisation of key analytes (BTEX and MTBE) could determine the specific fuel and so locate the particular leaking UST. In addition, the magnitude of the leak and an early indication of the general plume direction can be ascertained by following the MTBE contaminant plume. Monitoring specifically for MTBE may have significant advantages over other fractions present in petroleum fuels:

- MTBE is highly soluble and most European soils are ‘moist’ soils, with steady groundwater flow and frequent recharge (by precipitation). MTBE will therefore rapidly dissolve into soil water and will migrate downgradient from the source.
- MTBE is present in much higher volumes (>15 % v/v) than benzene (>2 % v/v) and has a significant impact on groundwater.
- BTEX is prone to adsorption by soils and the more soluble benzene fraction is rapidly degraded by indigenous soil micro-organisms. Dissolved-phase MTBE has a low tendency to adsorb to soil or to volatilise into soil vapour and is resistant to biodegradation.
- In moist or saturated soils, oxygenates may be readily detectable from soil samples (Nichols *et al.*, 2000). Alternatively, groundwater sampling tools, such as a Hydropunch® or Geoprobe®, can enable one-time sampling of groundwater to provide a ‘snap-shot’ of groundwater quality at a particular location.
- MTBE plumes attenuate much slower than BTEX and may continue to advance even after BTEX plumes have stabilised.

The overall goal of this work was to provide an integrated early warning/characterisation system for protection of groundwater at susceptible sites. This screening approach was supplemented by detailed laboratory-based evaluation/validation procedures, with the ultimate aim being to enable a reduction of manual site monitoring and characterisation costs. As MTBE contamination of groundwater supplies has now become a massive global problem, the second

part of this work has concentrated on the development of a novel MTBE immunoassay (IA) to enable rapid, accurate and inexpensive *on-site* analysis of water samples. The development of the novel ELISA specifically for MTBE, as described in this work, will enable on-site quantification for MTBE and will allow identification of the pollution source and MTBE migration patterns, thereby aiding the rapid implementation of appropriate remedial actions. The remaining sections of this chapter discuss the background and approaches to this development.

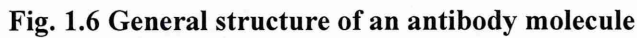
1.5 INTRODUCTION TO IMMUNOASSAYS

IA technology, the detection and measurement of target compounds using the specific binding characteristics of antibodies (Abs) is one of the most important techniques used in analytical cell and molecular biology. IAs are now widely applied in many scientific disciplines and are used to detect an extensive range of substances, including large and small organic and inorganic molecules, cells, cellular components and micro-organisms (Delves, 1995). They can be found in a wide variety of formats and are increasingly being developed and employed for environmental monitoring purposes, many having achieved US EPA acceptance for field-based analytical screening and site characterisation.

The following Sections (1.5.1-1.5.2) outline the general principles of IAs and the most common formats available, together with examples of environmental applications. Section 1.6 describes the IA developed specifically for the detection of the fuel oxygenate MTBE during this work.

1.5.1 General Principles of Immunoassays

The fundamental principle of IAs is that they utilise biologically generated immunoglobulin proteins – antibodies (Ab) which react with specific target compounds – antigens (Ag) to form antibody-antigen complexes (Ab-Ag). Abs are globulin proteins (immunoglobulins) and are produced in response to the presence of foreign molecules within the body). Whilst there are 5 classes of Abs: IgG, IgM, IgA, IgD, IgE, those employed in IAs usually belong to the IgG class, which is the predominant Ab in the secondary mammalian immune response and are produced *in vivo* by B-cells in conjunction with T-helper cells (Edwards, 1996). Native IgG antibodies have a molecular weight of approximately 150,000 Da., comprising of two 50,000 heavy and two 25,000 light polypeptide chains (Fig. 1.6).



1.5.1.1 Primary antibody (PAb)

Monoclonal Abs

20

the target Ag. The ideal monoclonal Abs (MAbs) will have a single epitope specificity (a site on an Ag that is recognised by an Ab) and limitless amounts of identical antibody can be supplied (through simple cell culturing techniques). Although costs and practical investments for MAb production are initially high, the producer has access to an effectively immortal cell line producing antibody of the desired specificity. This approach is now the preferred and established practice for IA design (Edwards, 1996).

Polyclonal Abs

Polyclonal Abs are heterogeneous Abs, derived from a number of different cells of the immune system after immunisation of a mammal with the target Ag. These are commonly obtained from mice, rabbits, goats or sheep. The polyclonal antiserum can be utilised at this stage, although the IgG fraction will contain many Abs of differing specificity's (Edwards, 1996).

1.5.1.2 Ag conjugation to carrier protein

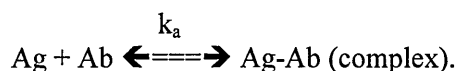
Most organic pollutants are molecularly too small, approximately <5000 Da. to induce an immunogenic response. In these cases, the molecules need to be conjugated to a carrier protein in order to provoke an immune response (Edwards, 1996). MTBE (88 Da.) is an extremely small molecule and is indeed classed as a 'hapten', which is not immunogenic by itself, but if linked to a much larger molecule (>30,000 Da.), such as a carrier protein, becomes immunogenic and can trigger the formation of complimentary antibody (Levinson & Jawetz, 1998). Theoretically, various immunogenic carriers can be used. The most common are: bovine serum albumin (BSA), human serum albumin (HSA), rabbit thyroglobin and keyhole limpet haemocyanin (KLH).

1.5.1.3 Binding Mechanisms of the Ab-Ag Complex

The binding of PAb to an antigen (Ag) is dependent on non-covalent, reversible interactions in equilibrium with free components. It depends upon three main factors:

1. The PAb-Ag bonds, via Van der Waals forces, electrostatic bonds, hydrogen bonds and hydrophobic interactions, which enables high selectivity.
2. The strength, or affinity, of these interactions
3. The stability, or avidity, of the immune complexes

The affinity is a measure of the strength of binding of an epitope (a binding site on the antigen) to an AB. Binding follows basic thermodynamic principles of any biomolecular interaction, as follows:



The affinity constant k_a is described as $k_a = [\text{Ab-Ag}] / [\text{Ab}] \cdot [\text{Ag}]$ where $[\text{Ab}]$ = concentration of Ab; $[\text{Ag}]$ = concentration of free Ag; and $[\text{Ab-Ag}]$ = concentration of the Ab-Ag complex.

This affinity constant k_a can be affected by temperature, pH and the composition of the aqueous medium. Changes in these factors can affect the amount of Ab-Ag complex formation at equilibrium. Any alteration in these factors may change the k_a and either drives the reaction further towards greater Ab-Ag complex formation; or release of the bound antigen. High affinity antibodies perform better in IA technologies and have the ability to bind antigen more effectively in a shorter period of time. This is due to the fact that the antibody reaction is diffusion controlled. In essence therefore, an increase in affinity equates to greater assay sensitivity.

The avidity is the measure of the overall stability of an Ab-Ag complex. The strength of any interaction is governed by three major factors: the intrinsic affinity of the antibody (for the epitope); the valency of the Ab and Ag and the geometric arrangement of the interacting components. Multivalent interactions increase the overall avidity of immunoassay procedures.

1.5.1.4 IA labelling systems and detection methods: An overview

Once the antibodies have been developed towards a particulate analyte, the choice of labelling (to recognise the Ab-Ag complex) are integral to successful assay design and applications. The binding of the Ab-Ag complex may be visualised using precipitation or agglutination techniques. A more applicable approach is to label the antibody (or the antigen) with a molecule that can be readily detected. The choice of label remains largely dependent on the specific characteristics and expected concentrations of the target analyte.

Radioisotope-labelled immunoassays (RIAs) were initially developed in the early 1970's, with the radioisotope ^{125}I most commonly used due to its high signal to noise ratio. The labelled Ab or Ag is detected by direct counting with a gamma counter or through autoradiography. RIAs can be highly sensitive, but require compliance with a number of stringent, regulatory controls and legislative restrictions, as well as requiring specialist laboratory areas (Delves, 1995).

However, a more sensitive approach is to use either an enzyme or fluorophore label, coupled to either streptavidin (for use with biotinylated Pabs), or to a secondary Ab (raised against the PAb). Such labels have become increasingly popular in modern IA applications. Enzymes

(commonly horseradish peroxidase, HRP) can be selected to convert non-coloured colorimetric substrates (chromogen) to coloured products, such as 3,3',5,5'-Tetramethylbenzidine (TMB) or 2,2'-Azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS). Photoluminescent compounds in immunoassays (FIAs) can provide even greater sensitivity than colorimetric substrates. Fluorescein isothiocyanate (FITC) is usually the label of choice for immunofluorescent IAs, as coupling procedures are straightforward works with almost all antibodies. Additional labels commonly used include tetramethylrhodamine isothiocyanate (TRITC), Texas Red (TR) or phycoerythrin (PE). Detection is achieved through either a fluorescence microscope or a flow cytometer (FC). A wide range of fluorimeters are commercially available, which may use cells, tubes or flow cells as sample holders, as well as dedicated 96-well plate readers (Blincko, 1996). In general, the choice of fluorochrome is determined by the availability of the laser and optical filter systems (Delves, 1995).

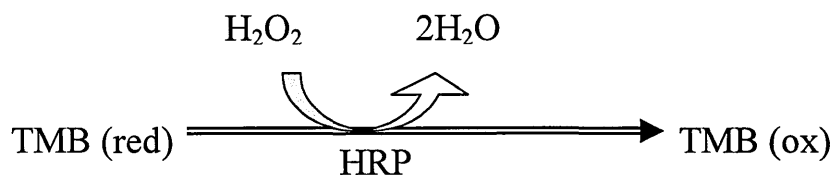
1.5.2 Enzyme-linked immunosorbent assays (ELISAs)

As all IAs are based on the selectivity and sensitivity of the Ab-Ag reaction, due to the wide variation in Ab-Ag interactions, each IA has to be specifically developed for the target of interest - the antigen (Ag). It is not realistically feasible to discuss the gamut of IA formats available and so a selective overview has been provided, which has a direct relationship to the MTBE IA developed in this work. Tijssen (1993) provides an excellent review on many enzyme-based IA methods. Enzyme-linked immunosorbent assays (ELISAs) are one of the most common IA techniques used for testing large numbers of samples, and quantitative results can be obtained by comparisons against known concentrations of control Ags. ELISA methods combine the specificity of PABs with the amplification of Ab-Ag interactions by enzyme catalysis. Specific enzymes, such as HRP, or alkaline phosphatase, are conjugated to either Ag or Ab and through enzymatic actions on a colour producing agent (chromogen), enables both qualitative and quantitative analysis and are often capable of detecting very low amounts of analyte (ppb).

The colorimetric approach using HRP enzyme with the chromogen TMB was selected for the development of the MTBE immunoassay and the catalytic reaction is described as follows:

The oxidation of the chromogen TMB is catalysed by the enzyme HRP, which concomitantly reduces H_2O_2 to $2\text{H}_2\text{O}$ - oxidised TMB produces a blue colour and absorbance can be read at 630 nm. The colour reaction can be stopped with the addition of sulphuric acid and the colour changes to yellow, absorbance can then be read at 450 nm. TMB is very sensitive, requiring less

material to obtain the same sensitivity as obtained with ABTS and is oxidised more rapidly than other HRP substrates, resulting in faster colour development (Torrance, 1998).



The majority of ELISAs are performed in the wells of microtitre plates in which reactants are immobilised and there are two main formats: direct assays employ specific PAb conjugated to an enzyme, whereas indirect assays, the Ag-specific PAb (IgG) is in turn detected by secondary Ab-anti-IgG enzyme conjugate, of which a wide range are commercially available. ELISAs can be readily standardised and optimised to minimise variation between and within assays. These developed protocols can then be easily followed by individuals with little previous knowledge or training and so are ELISAs are extremely suitable for use as an ‘on-site’ analysis tool. The MTBE ELISA developed in this work was a competitive ELISA, originally developed as a 96-well plate format. Specific details regarding the initial development of the MTBE ELISA is provided in Section 1.6.

1.5.2.1 Environmental applications of ELISAs

The US EPA have official, approved ELISA methodologies for the determination of total petroleum hydrocarbons (TPH, Method #4030) and polynuclear aromatic hydrocarbons (PAHs & BTEX Method #4035), as well as other methods for certain pesticides (Castillo *et al.*, 1998). ‘RaPID’ ELISA test kits are commercially available for a wide range of analytes, which provide a relatively inexpensive, rapid (<2 hr), sensitive screening method for analyte detection, commonly in aqueous and soil matrices. These tests are rapid, sensitive, low-cost and simple field-test methods. Castillo *et al.*, (1998) evaluated the commercial SDI ELISA kits for determining pentachlorophenol, carcinogenic PAHs and BTEX compounds within industrial effluents, in comparison to LC/MS detection. The protocols described were validated as providing accurate and sensitive identification of the target analytes (Castillo *et al.*, 1998; Richardson, 2000). Indeed, the commercial ‘Total-BTEX RaPID Assay’ kit (Strategic Diagnostic Inc., Newark, USA) conforms to the US EPA’s Method #4031 (SW846 Manual of Methods). The RaPID assay kit was used within this work and is so described in further detail.

1.5.2.2 Measurement of BTEX compounds by IA

The 'Total-BTEX RaPID Assay' kit (SDI, Newark, USA) was chosen for its broad antibody cross-reactivity towards BTEX compounds and its general availability. Field test screening for BTEX compounds by immunoassay has also been accepted by the US EPA (Method #4035) for use as a semi-quantitative analytical tool. After the sub-surface sensors (Chapter 2) had been triggered through fuel contamination, experiments were conducted on fuel-contaminated Mk 3 sensor membranes (Chapter 4) to determine if the BTEX contaminants could be extracted from the membrane matrices and measured. Should BTEX extraction (and subsequent quantification) from contaminated sensor membranes prove successful, the sensors could be considered as a semi-analytical tool, as contaminants such as petrol (containing BTEX and fuel oxygenates) and diesel (lacking fuel oxygenates) or other oils can be distinguished.

The Total-BTEX RaPID Assay kit (Fig. 1.7) applies the principles of a competitive ELISA and can measure in the low ppm range (0.9-3 ppm). The format of the RaPID immunoassay is based on using PAb bound to magnetic particles. This is added to water samples (suspected of contamination) and with the addition of an enzyme-labelled (HRP) BTEX conjugate, any free BTEX within the water sample competes for the PAb binding sites. Bound BTEX (free or conjugate) to the PAb on the magnetic particles are retained in the sample tubes during the washing steps through the addition of a magnetic plate. This holds the magnetic particles tightly against the base of the tubes. A proprietary TMB-based proprietary colour substrate is then added and the resulting colour is measured using a portable photometer (Fig. 1.7).



Fig. 1.7 The Total BTEX RaPID field test kit

From l-r: groundwater sample, portable photometer, RaPID Assay kit (as supplied), sample rack and magnetic plate (placed on the base of the sample rack).

BTEX data interpretation

Supplied BTEX standards were tested and the calculated % B/B_0 plotted on a logit ordinate (y axis) on a logarithmic abscissa (x axis) to obtain a linear calibration curve. The % B/B_0 of unknown samples were then plotted against this and the BTEX concentration determined by reading off the x-axis. The BTEX immunoassay data was reported by the assay signal obtained for a given sample (B) as a percentage of the assay signal of the zero diluent or blank (B_0), therefore giving % B/B_0 . As the assay signal was inversely proportional to the amount of

analyte in the sample (darker colour with higher absorbance indicated lower analyte concentration), the % B/B₀ value decreased as the concentration of sample analyte increased.

1.6 DEVELOPMENT OF THE MTBE IMMUNOASSAY

As there was no commercially available antibody (Ab) for MTBE and/or other fuel oxygenates, such as ETBE, TAME, DIPE or TBA, an important aspect of this work was the development of primary antibody (PAb) raised against MTBE. Correspondingly, extensive development and evaluation of an immunoassay (ELISA) was required for the quantitative measurement of MTBE in soil and water samples. The goal of this work was to provide an integrated early warning/characterisation system for protection of groundwater at susceptible sites. This screening approach (of water samples) was supplemented by detailed laboratory-based evaluation/validation procedures, with the ultimate aim being to enable a reduction of manual site monitoring and characterisation costs. The following Sections relate directly to the immuno-reagents and IA techniques developed within this work for the development of the MTBE ELISA.

1.6.1 MTBE ELISA Reagents

1.6.1.1 Ag conjugates

BSA was the chosen Ag carrier protein in this work and both MTBE (88 Da.) and a similar compound, 7' Methoxy-3,7-dimethyloctanal (MODMO, ~186 Da.) were conjugated to this carrier (Diaclone SAS, Besançon, France). The novel aspect of the BSA-MODMO conjugate was that one end of the MODMO structure was an active aldehyde group, suitable for binding (by Schiff base formation) to the amine groups present on the BSA protein; the other end had a group structurally analogous to MTBE (Chapter 6, Fig. 6.34).

For this work, the suppliers (Diaclone SAS) reported that the BSA-MTBE Ag produced no immunogenic response in their initial screenings. This was most probably due to the very small molecular size of MTBE resulting in lack of specific epitope recognition by the host species. The comparative large globular structure of the BSA carrier protein possibly masked the conjugated MTBE structures, resulting in steric hinderance effects. Alternatively, BSA-MTBE conjugations may have been unsuccessful due to the chemistries involved.

The BSA-MODMO Ag conjugate had produced immunogenic responses and after extensive hybridoma preparation and screening (of ~3000 hybridomas), a single monoclonal PAb exhibited binding affinity to the BSA-MODMO Ag conjugate. This was supplied by Diaclone

SAS for development of the MTBE ELISA. Two forms of the mouse monoclonal IgG PAb were supplied and subsequently evaluated in this work. One was native PAb and the other form was the mouse IgG PAb, which had been conjugated to biotin.

1.6.1.2 Primary Ab (PAb)

Monoclonal PAbs were raised against BSA-MTBE and BSA-MODMO conjugates (Diacclone SAS, Besançon, France). Two forms were then supplied: native PAb (unconjugated) and PAb conjugated (labelled) to biotin (1.6.1.3). These two PAb forms were then used for the development of the 96-well plate ELISA (Fig. 1.9), followed by extensive evaluations, after which the native PAb was then selected for use in later adaptations (1.6.2-1.6.3).

1.6.1.3 Labelling systems employed for the MTBE ELISA

1.6.1.3.1 Biotin/streptavidin-HRP labelling

One of the most common techniques is to use the biotin/streptavidin combination, in which the streptavidin has been labelled with the enzyme horse radish peroxidase (HRP), which is the most common and versatile label for Abs in immunochemistry, particularly for Western blotting and ELISA. However, the choice of label remains largely dependent on the specific characteristics and expected concentrations of the target analyte, as well as personal preference. Biotin is a small molecule (244 Da.) that is tightly bound by the proteins ‘avidin’ (from egg white) and ‘streptavidin’ (from *Streptomyces avidinii*). Each tetrameric molecule of avidin or streptavidin (66 & 58 kDa. respectively) is capable of binding four molecules of biotin. Many biotin molecules can be coupled to a protein, enabling the biotinylated protein to bind more than one molecule of streptavidin. The exact number of biotin molecules bound to the PAb was proprietary to the suppliers (Diacclone SAS).

The biotinylated PAb is incubated with the sample (containing analyte) and then a second IA incubation is made with the streptavidin-HRP (s-HRP). A chromogenic substrate, such as (TMB) is then used to react with the HRP enzyme and yields a soluble, coloured product, of which the absorbance (A) is determined. This method was employed for the development of the MTBE ELISA using the biotinylated PAb.

1.6.1.3.2 Labelling with secondary antibody-HRP conjugate (2Ab-HRP)

After probing the sample with native PAb, detection of any bound PAb can be made with a secondary antibody, coupled to a label (most usually HRP). If the PAb is a mouse IgG monoclonal Ig, then the 2Ab will be Abs raised against mouse IgG. The 2Ab is usually (but not always) a polyclonal raised against total Igs from the target species, and has to be raised in a

different species, commonly either goat or chicken. The 2Ab-HRP used in the development of the MTBE ELISA employing the native PAb was a commercial polyclonal anti-monoclonal mouse IgG goat IgG polyclonal, conjugated to HRP).

1.6.2 Principle of the competitive MTBE ELISA

As previously mentioned, ELISAs exploit the specific binding interaction between the target analyte (Ag) and primary Ab (PAb) enabling the quantification of compounds through signal amplification using an associated enzyme label. During this work, this was either streptavidin-HRP (using biotinylated PAb) or 2Ab-HRP (using native PAb). In both cases, the colour substrate used for the enzymatic catalytic reaction was TMB. The novel MTBE ELISA developed in this work employs a direct, competitive assay format, wherein a protein–Ag (coating Ag) conjugate is first immobilised onto a solid support, such as a microtitre well wall. Once an Ab preparation is added to the immobilised antigen analogue, free MTBE Ag present in the sample competes with the immobilised (coating) Ag for PAb binding sites (Fig. 1.8).

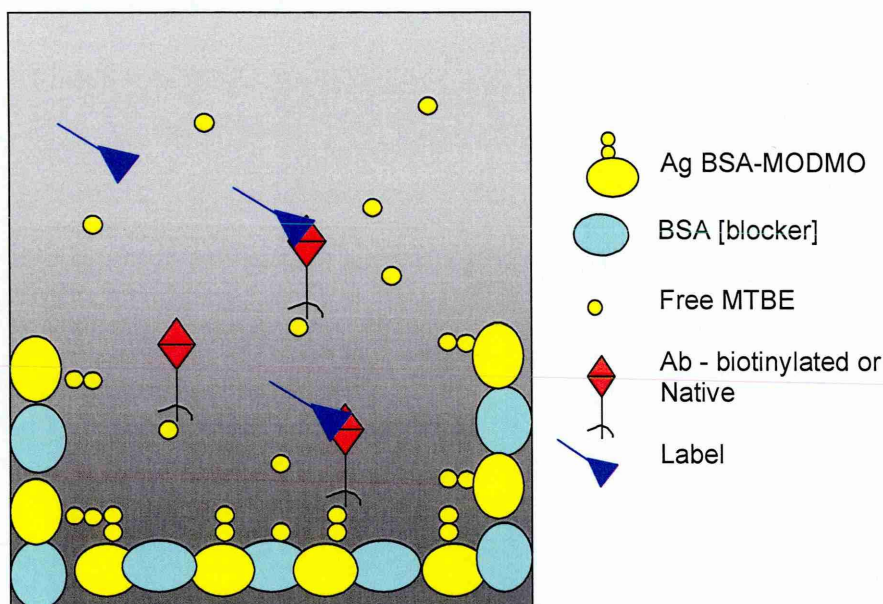


Fig. 1.8 Principle of the MTBE competitive ELISA.

BSA-MODMO coating buffer is immobilised to the microtitre well walls and residual non-specific binding sites blocked with BSA protein. Free MTBE and PAb (either in native form or conjugated to biotin) is added to the well. Free MTBE and immobilised MODMO compete for PAb binding sites. After incubation, the well is washed to remove residual free MTBE and MTBE-PAb complexes. HRP enzyme label is then added, either conjugated to anti-mouse 2Ab-HRP, to bind to the native PAb (mouse IgG), or conjugated to s-HRP, which binds tightly to biotin. Excess label is washed from the wells and the TMB substrate added. Coloured enzymatic product is then measured after a pre-defined incubation time.

The binding affinity of the PAb is directed towards both free and immobilised MTBE. In simple terms, the lower the MTBE concentration (free MTBE) in the sample solution, the greater the proportion of PAb binding to immobilised Ag. On washing the microtitre wells, free anti-MTBE PAb is displaced from the system. A PAb or biotin-specific tracer protein (coupled to HRP) is then added to the system.

Following binding and washing, residual enzyme activity, which is inversely proportional to the original free MTBE concentration, is determined by adding enzyme substrate (TMB) and the absorbance (A) of the coloured enzymatic product determined after a suitable period of colour development. Conversely, elevated concentrations of free MTBE in solution will result in a depressed assay signal. Sensitivity of any ELISA system depends on the affinity of the PAb for the Ag, the enzyme label and the choice of colour substrate.

1.6.3 Initial protocol for the 96-well plate MTBE ELISA

Monoclonal PAbs were raised against BSA-MTBE and BSA-MODMO conjugates (Diacclone SAS, Besançon, France). Two forms were then supplied: native PAb and PAb labelled with biotin. These two PAb forms were then used for the development of the 96-well plate ELISA (Fig. 1.9), followed by extensive evaluations, after which the native PAb was then selected for use in later adaptations (1.6.5-1.6.6).

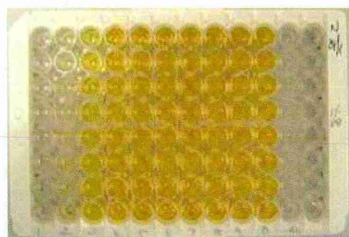


Fig. 1.9 Standard 96-well plate ELISA

A 96-well magnetic plate. The figure shows the results of a typical MTBE ELISA assay, with decreasing amounts of MTBE concentrations (l-r). The increase in colour seen is inversely proportional to the MTBE concentration. The end two rows (r) were used as controls (no PAb/no Ag).

The initial starting IA protocol was devised by using the most common commercial ELISA methods, which are generally considered to be standard practice at the beginning of any ELISA development stage. The initial concentrations of Ag and PAbs used for early evaluations were those suggested by the respective reagent suppliers. Once the BSA-Ag antigen had been immobilised on the 96-well surface (Fig. 1.8), wells were incubated with a solution of blocking protein, bovine serum albumin (BSA), in order to block surplus protein binding sites and prevent non-specific binding. An alternative blocking buffer (BB) is casein (milk powder), although there are now many more alternatives commercially available. A range of blocking

buffers were examined (Chapter 5). Detergent, such as Tween 20 is also commonly employed, either with the blocking protein solution or within the washing buffer (WB). Full details of the experimental factors employed during this work is reported in Chapters 5 and 6.

1.6.4 Alternative protein conjugations

An alternative labelling approach was to directly label the PAb with an enzyme, such as HRP, that could be readily detected (once bound to the Ag-coated wells) simply by the addition of a chromogen (TMB). This would have changed the assay into a more direct assay format and thus not require a secondary binding step (such as from s-HRP or 2Ab-HRP), thus reducing overall assay time. Additional benefits of labelling a primary IgG Ab would be to reduce the amount of background signal and have less non-specific binding (NSB). This has been achieved commercially and kits are available for fluorescent-labelling PAb, for example, the Alexa Fluoro Monoclonal and the Zenon IgG Antibody-labelling kits (Invitrogen Ltd., Paisley, UK). An example of PAb-HRP direct labelling is shown in Fig. 1.10.

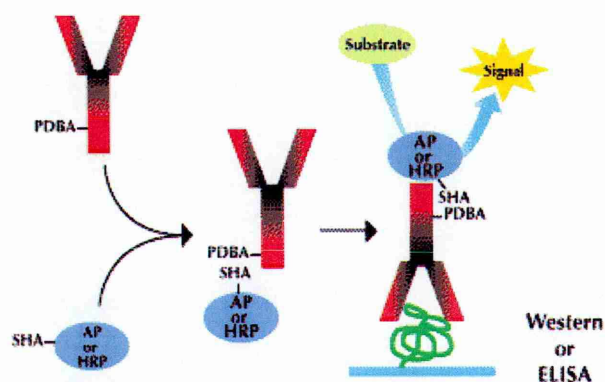


Fig. 1.10 Theoretical example of PAb-labelled HRP

The primary antibody (PAb) has been modified with phenyldiboronic acid (PDBA) in order to enhance binding properties to the HRP (or alkaline phosphatase, AP). Once the labelled PAb-HRP has bound to the immobilised Ag, a chromogen (TMB) can be added, which will be oxidised by the HRP to give a signal. (Source: Versalinx®, Cambrex Bio Science Ltd., Nottingham, UK).

A second alternative was to attempt to directly conjugate HRP to the antigens; MODMO and BSA-MODMO, by linking the active aldehyde end of MODMO directly to HRP by a Schiff's base reaction, which was also attempted during this work and is reported in Chapters 5 and 6.

1.6.5 Transposing the microtitre plate ELISA into an ELIFA format

1.6.5.1 Environmental applications: Immuno-flow through assays (IFAs)

Immuno-flow through assays (IFAs) are more recent IA-based technologies which have been developed for a number of environmental applications. For example, the explosives 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) can be detected to 0.1-0.5 ng/ml concentrations in groundwater by displacement flow IFA, where microcapillary flow-cells are coated with Ab and a fluorophore-labelled Ag is then bound to the coating Ab. The labelled Ag is then displaced by target Ag in the sample and can be measured by a portable spectrofluorometer (Narang *et al.*, 1998). An commercial IFA assay (Hydrofluor™ Combo kit (SDI Inc., USA) has been developed that can simultaneously detect *Giardia* and *Cryptosporidium* antigens (by using a combination of Ag-specific Abs) in water samples (Torrance, 1998).

1.6.5.2 Enzyme-linked immuno-flow through assays (ELIFA)

An alternative to using fluorophore labels is to again use enzymes, such as HRP, in conjunction with a chromogen to produce a signal, as for ELISA. An ELIFA system takes advantage of the ability of solutions to pass through porous solid phases, such as a membrane, on which the antigen is immobilised and where the immuno-reaction occurs. In principle, the assay works in the same way as an ELISA except that the solutions containing antigen, blocker, primary and secondary antibody are, in sequence, pulled through the porous solid phase (membrane). Ultimately, signal produced by labelled antibody allows quantification of the target antigen.

One of the advantages of the ELIFA is that the passage of solutions through the membrane pores increases the rate of interactions between Ab and Ag, whereas in a 96-well based ELISA, interactions are likely to be more diffusion-limited (even with plate-shaking). A consequence of the much higher reagent-mixing efficiency of the ELIFA can be that a typical ELISA requires a period of time of 18-20 hours to be completed whereas an ELIFA can take only 30 to 60 min (Hermanson *et al.*, 1992).

Pierce (PerbioScience UK Ltd.) developed a generic Easy-Titer™ ELIFA System, which sandwiches a nitrocellulose membrane between a 96-well sample application plate and a vacuum chamber. This system follows the standard principles behind an ELISA, using Ag, monoclonal Abs and secondary labelled Abs can be immobilised onto the membrane by using the specific analyte and detected using an appropriate chromogen. The resulting colour can then be measured using a microtitre plate reader. A competitive membrane-based 'dipstick' ELIFA

test has also been developed for the rapid testing for atrazine in water samples with visual detection limits of 10 µg/l (Gabaldón *et al.*, 2002).

The optimised 96-well microtitre plate-based MTBE ELISA was therefore transposed firstly into an ELIFA using a membrane as the porous solid support phase, held within a flow cell.

1.6.5.3 ELIFA flow cells

The apparatus used to transpose the MTBE ELISA into an ELIFA protocol consisted of purpose-designed flow cells (John Bolbot, Cranfield University) containing a small membrane disc. Each flow cell is formed by two cylindrical components identified as the top and the base part (Fig. 1.11). The base part has on its upper face a circular cavity which accommodates the membrane; the top part is then tightly assembled on its top with 3 screws, thus the two components firmly hold the membrane (Fig. 1.12).

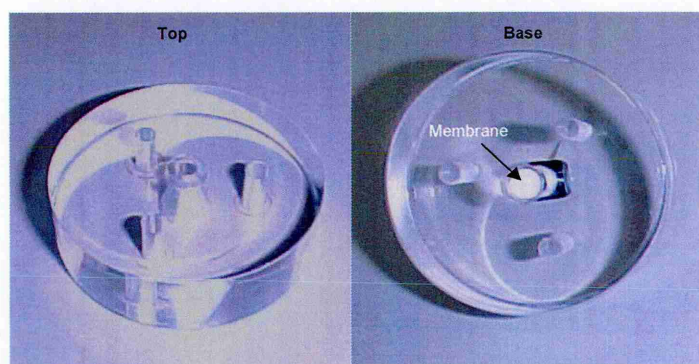


Fig. 1.11 Components of flow cell.

The flow cell consists of two components: The top part of the cell contains a central loading chamber for reagent solutions. The base section has a central recess for the membrane disc. Underneath this, there is small reaction chamber with a cannula connecting the cell to a peristaltic pump. Both are tightly assembled via 3 fixing screws.

The top part has a funnelled 'loading chamber' into which the reagents are loaded; this can contain a maximum volume of 750 µl. The base part is provided on its lower side with a cannula that collects the solution pulled through the membrane and which is connected to a peristaltic pump. Suction produced by the pump pulls reagent solutions through the membrane. Reagents are loaded on the top surface of the membrane and aspirated through this by the pump, all reagents are discharged into a waste bottle except the coloured solution generated by the TMB reaction with HRP which is collected in a well of a microplate and then read on a plate reader. It is important to emphasise that the number of assays that could be run simultaneously was limited to two as only two cells were available and the peristaltic pump used was also limited to two channels (tubes). To perform simultaneous assays, it was necessary to prepare the

two flow cells with the respective membranes and to load the reagent solutions into the loading chambers at a precise time and to then progress both assays at the same rate. Furthermore, it was important that the solution loaded into each flow cell had completely passed through the membrane before the next solution was added so that the former did not dilute the following reagent. However, it was also important that the membrane did not get dry. The ELIFA procedure therefore required the constant presence of the operator.

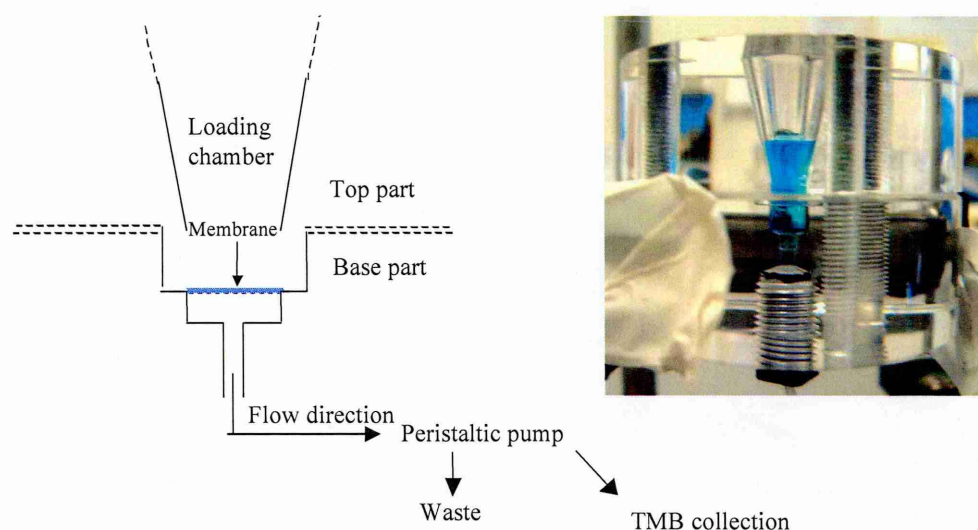


Fig. 1.12 Schematic representation of the section a flow cell.

The top and base parts clamp the membrane around its perimeter. Solutions are pipetted on the top of the membrane in the 'loading chamber', a peristaltic pump pulls them through. The insert on the right shows a working MTBE ELIFA assay in the assembled cell. The blue solution in the loading/reaction chamber is through the action of HRP on the TMB colour substrate. With continuous pumping, only colour develops in the lower chamber. In this example, the colour reagent had been flushed back and forth through the membrane, producing colour in both chambers.

1.6.5.4 Choice of membrane

The flow cells were designed to use membrane discs as the porous solid phase. Membranes are large three-dimensional sheets constituting by a framework that delimits a net of interconnected channels the size of which can usually be controlled in manufacture.

The chemical composition of membranes varies greatly; the most common are polymers of cellulose, polyamide (nylon) and also polyvinylidene difluoride, polysulfone and many more. The most popular for biological applications are probably the nylon and the cellulose membranes; many of these have also been chemically derivatised with a large number of different functional groups which modify their surface characteristics. Membranes with a small pore size are classically used to separate mixtures of different molecular sizes; this is the principle of size-exclusion filtration, which represents the main application of membranes in the

scientific field. However, in recent years the interest of the analytical bio-scientist has moved toward the potential application of membranes as the solid phase for quantitative immuno-diagnostics and the field is still relatively unexplored, only a few reports have appeared in the literature.

The nature of the membrane used in the ELIFA was expected to be an important factor affecting protein immobilisation and therefore assay performance. Four membranes; Biodyne+, Hybond+ Nylon, Immunodyne and DEAE+ cellulose were evaluated and compared. With the exception of Immunodyne, these membranes were derivatised to carry a positive surface charge. Positive-charged membranes were selected as at pH 7.4 the BSA-Ag used in the ELIFA carries a strong negative charge. By using a positively-charged membrane, subsequent electrostatic interactions were envisaged between the proteins and the membranes, rather than the usual hydrophobic interactions involved in membrane immobilisation. A higher protein binding and (therefore) subsequent signal size was envisaged. In contrast, Immunodyne is a neutral membrane, with a proprietary latent chemistry, which forms covalent bonds with amines and therefore binds proteins non-specifically. It is activated by wetting, and so was stored under nitrogen until use.

Isoelectric point (pI) of proteins

The isoelectric point (pI) is the pH at which a protein has a net charge of zero. Proteins are 3-dimensional polymers of amino acids, which have side chains with ionisable groups, the pI of a protein is consequently determined by its amino acid composition and to some extent, its shape. The positive and negative charges on a protein balance exactly at its isoelectric point. Knowing the pI of a protein is useful for developing buffer systems.

Proteins generally have a minimum solubility when the pH of the solution is close to the protein's pI. Therefore, pI information is used to maximise protein solubility or to precipitate the protein from the solution. BSA has a pI of ~4.5, therefore it would be electrostatically neutral at pH 4.5. At a more alkaline pH of 7.4 (as used in the ELISA and ELIFA), the H⁺ ions would have dissociated to leave the protein with a net negative charge. Thus, selecting positively charged membranes (Biodyne+, Hybond+ Nylon & DE81+ cellulose) should have optimised BSA-Ag binding (electrostatic) to the membrane surface. The important point is that, on the alkaline side of their pI, proteins are negative, whereas on the acid side they are positive.

1.6.5.5 The ELIFA protocol design

ELIFA work described by Faedda (2003) in association with the optimised ELISA assay formats were combined and adapted (as described in Chapter 5) to set up a starting protocol

which was then worked up and optimised. Full methodologies and subsequent results are given in Chapters 5 & 6.

1.6.6 Transposing the 96-well MTBE ELISA into a magnetic bead format

1.6.6.1 Applications: Magnetic-bead based immunoassays

Magnetic-bead based IAs have traditionally been developed for medical purposes, rather than environmental applications, primarily due to their low-cost and ‘ease of use’ advantages over expensive, sophisticated medical technologies. A prime example is the development of a number of magnetic bead ELISAs for the detection of CD4 lymphocytes in blood samples to determine human HIV infection. The reference standard for CD4 cell testing is usually by molecular-based reverse-transcriptase polymerase chain reaction (RT-PCR), which is a highly technical, complex technique, which requires highly trained personnel and expensive equipment (Crowe *et al.*, 2003). The Capcellia CD4/CD8 Whole Blood ELISA and the TRAX CD4 Test Kit both use magnetic Dynabeads™. The Capcellia system uses monoclonal anti-CD4 monoclonal PABs coated onto the magnetic beads, with detection by secondary capture from a second monoclonal ‘anti-CD4 monoclonal PAB’ coupled to peroxidase. The TRAX CD4 ELISA Test Kit uses HRP-labelled monoclonal anti-CD4 Pabs. Both systems use a 96-well plate format (Crowe *et al.*, 2003).

Magnetic-bead based IAs have also been developed for environmental applications, particular for analysing water samples. Magnetic beads coated with monoclonal PABs specific for the bacterial flagellae have been used to isolate *Pseudomonads* from samples of unconcentrated lake water (this technique is sometimes referred to as an immuno-magnetic separation, IMS, assay). Similarly, magnetic Dynabeads coated with anti-*Cryptosporidium* Abs are now commercially available (Dynabeads® anti-*Cryptosporidium*, Dynal Biotech, Wirral, UK) for separating oocysts from concentrated water samples prior to further analysis by IFA (Torrance, 1998). Other Dynabead-based immunoassays have also been used for the certified laboratory-based detection and separation of the common food and water pathogens *E. Coli* 0157 and *Salmonella* (Warburton, (1997). Once separation is achieved by binding to the Ab-coated beads, the bead-bacteria complexes are then plated onto selective culture media and the colonies grown and examined for their specific characteristics.

The commercial Total-BTEX RaPID Assay Test kit (1.5.2.2), as used during this work for measuring BTEX in water samples, also incorporates magnetic beads as a protein support

substrate. As this system is proprietary to SDI, the commercially available DynabeadsTM were selected in order to also transpose the 96-well plate MTBE ELISA into a tube-based, magnetic bead format.

1.6.6.1.1 'Dynabeads'

Dynabeads® were originally developed for use as a gentle and efficient protein separation method (DynaL Biotech, Wirral, UK). The spherical, polystyrene magnetic beads (2.8 µm dia.) contain iron (Fe) and are surface-coated with a polyurethane layer (Fig. 1.13). This has an active chemistry (Fig. 1.14) which can be used to immobilise protein ligands with specific affinity for the required target (cells, nucleic acids, proteins). The beads will consequently selectively bind the desired target when added to a heterogeneous suspension. The resulting target-bead complex is then removed from the suspension using a magnet (Fig. 1.15), as the beads are drawn to the side of the tube facing the magnet. The supernatant is then removed by pipette. This method allows easy washing, gentle separation and concentration of the target without the need for centrifugation or expensive separation columns.

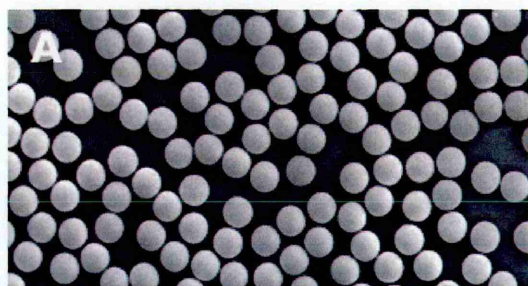
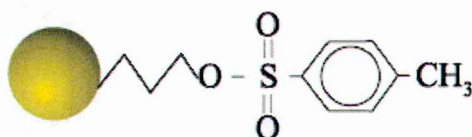
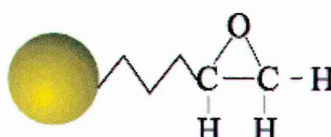


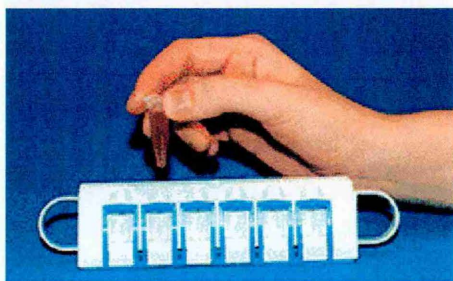
Fig. 1.13 Structural image of the Dynal magnetic beads

Spherical (2.8 µm dia.) uniform (CV <3%) superparamagnetic Dynabeads exhibit magnetic properties only when placed in a magnetic field, with no residual magnetism. Beads are coated with a polymer shell to avoid toxic exposure to Fe. The spherical shape and defined surface chemistry minimises chemical agglutination and NSB, whilst giving optimal accessibility & reaction kinetics for rapid & efficient binding.

Two types of surface-activated magnetic beads were available and considered suitable for the ELISA, in particular for strong binding of the BSA-Ag conjugate. These were the Tosylactivated and Epoxy beads. The Tosylactivated beads (T-beads) were selected for the initial development work due to the more active surface chemistry (Fig. 1.14) and results are reported in Chapter 6. The Epoxy beads were also examined but initial conjugations proved unsuccessful; this work has not been reported in this thesis.

Surface tosyl groups**Surface epoxy groups****Fig 1.14 Surface chemistries of the Tosylactivated and Epoxy magnetic beads**

The polyurethane bead surface is activated by p-toluenesulphhydryl chloride to provide reactive tosyl groups for covalent binding of proteins containing primary amino or sulphhydryl groups. Epoxy (glycidyl ether) groups covalently bind protein amino groups. The Tosylactivated & Epoxy beads have the same hydrophobicity and bind the Fc part of Abs, both directly couple to proteins (including BSA) by covalent binding.

**Fig. 1.15 The Dynal MPC® magnetic rack**

The rack consists of a six tube rack, with a magnetic strip, which, when in place, holds the magnetic beads to the sides of the Eppendorfs. The magnetic strip can be removed for washing/mixing steps, leaving the tubes in the rack.

This concludes the introduction to this work. Chapters 2, 3 and 4 relate specifically to the design and development of the sub-surface sensors. The development of the MTBE ELISA and alternative formats is provided in Chapters 5 and 6, followed by overall conclusions and recommendations for future work (Chapter 7). A list of patents, proceedings and publications made during this work is given in Appendix 4.

CHAPTER 2

CHRONOLOGICAL DEVELOPMENT OF A SUB-SURFACE SENSOR-BASED UST MONITORING SYSTEM

2.1 INTRODUCTION

The environmental problems posed by leaking petroleum storage tanks have been described in Chapter 1. Current methods of monitoring such leaks have also been discussed (1.2) and these are of two principle types: (1) internal tank gauges which measure the amount held in the tank; or (2) routine manual sampling of soil around the site and/or sampling of groundwater from boreholes constructed around the area of interest. Detection of contaminating hydrocarbons in either soil or water matrices generally involves such samples being collected, transported and analysed in a laboratory, most commonly by gas chromatography (GC) or GC-MS (mass spectroscopy). US EPA legislation currently requires monthly monitoring of all USTs using such methods, which must be capable of detecting a minimum leak rate of 0.2 gallons per hour.

This chapter details the chronological development of the sub-surface monitoring system developed within this work. The chapter is divided into two main parts: The first part (Sections 2.2-2.5) begins with a reappraisal of existing sensor technology, which greatly contributed to the design of the Mk 1 sub-surface sensor (Section 2.3). Sections 2.4-2.5 describe the chronological development of the Mk 1-Mk 4 sub-surface sensors, which was influenced by the experimental work described in Chapter 4. The second part of this chapter (Sections 2.6-2.7) describes the simultaneous development of two novel data acquisition technologies which were essential for this work. A chronological summary of this work is provided in Table 2.1.

2.2 REAPPRAISAL OF EXISTING SENSOR TECHNOLOGY

A prototype reflectometric sub-surface sensing device for qualitative detection of liquid hydrocarbons was first developed by Ritchie *et al.*, (2000a). The basic principle behind optical reflectometry is the measurement of the intensity of a light beam reflected back from a surface. The device consisted of a simple 5 V optical circuit, with a current-limited photodiode, which provided a near infrared (NIR) light source directed at a glass-sensing window above it. A soil sample (held within a petri dish) would be placed on top. Reflected light from the sample would then pass back through the sensing window to be received by a separate phototransistor. Should the sample contain hydrocarbons, these would absorb a portion of the NIR radiation, resulting in a reduction of the intensity of reflected light. By measuring this reflectance (as an output voltage - V), loss of intensity was attributed to absorption of light by hydrocarbons present within the

Table 2.1 Chronological summary of the sub-surface fuel sensor development

The chronological redesign and development of the sub-surface fuel leak sensors from Mk1-Mk4 is shown. Brief summaries of the problems found and the subsequent actions taken as a result of the experimental work undertaken (Chapters 3 & 4) are highlighted, with corresponding results sections provided.

Sub-surface sensor model	Problem	Action	Related results
Cable oil sensor (NGT)	Too large. Large membrane diameter with relatively low sensitivity to fuel contamination. Use restricted - sensor proprietary to NGC under license.	Mk 1 design & manufacture	Sections 2.2-2.4
Mk 1	No existing technology available for data acquisition.	MedSci * data logging unit obtained for development with sub-surface sensors as complete monitoring unit	Section 2.6.2
	Each Mk 1 sensor drawing ~60 mA current draining battery power when inactive	Electronics modified to limit current demand to ~10-15 mA when sensor inactive in Mk 2 sensors.	Section 2.5.1 Results 4.3.1
	No software available for real-time evaluation of sensors	Development of a lab-based data acquisition interface and software programming	Section 2.6.1
Mk 2	Membrane too far back in head assembly. Free space prone to clogging with soil. General insensitivity to soil and water contamination.	Tapered head re-machined for Mk 3 sensor. Head assembly 'lip' reduced to absolute minimum. Overall head length reduced by 5 mm so mesh and O-ring can be discarded.	Section 2.5.1-2.5.2 Results 4.3.1
	Further improve sensitivity of sensor	Valuation of range of suitable hydrocarbon membranes and resistance to biofouling.	Results 4.2.3, 4.2.6
Mk 3	Glass sensing window deteriorates around edges through repeated tightening. Cable exit sealant deteriorates with repeated fuel exposure. Unable to repair electronics due to resin.	Extensive redesign of sensor head and body. Head length reduced. Sensor body incorporates brass 'lip' to protect glass. Additional O-ring seals on body enables electronic modifications. Reinforced cable exit design.	Section 2.5.3-2.5.4 Results 4.3.2, 4.4
Mk 4	N/A	N/A	Section 2.5.4 Results 4.4

* Medsci consultants Ltd. (Harpenden, UK).

soil sample. A simple reflectometric circuit diagram illustrating this principle is shown in Fig. 2.1, a prototype device based on this principle is shown in Fig. 2.2.

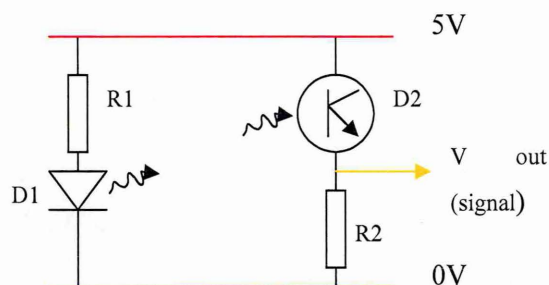


Fig. 2.1 Simplified reflectometric circuit diagram

Near infrared light (950 nm) was emitted by a photodiode (D1). Reflected light from the sample was detected by a phototransistor (D2) and measured as a signal voltage (V_{out}). When light is absorbed by hydrocarbons, there is a decrease in radiation incident upon D2 and a corresponding drop in signal (V) is recorded.

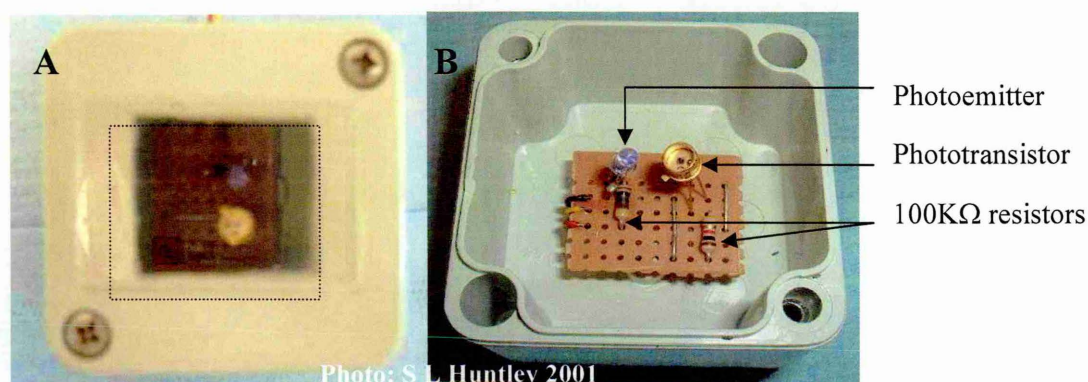


Fig. 2.2 Prototype reflectometric sensor

A) The complete sensor unit (Ritchie *et al.*, 2000a). The membrane was placed into the sampling vessel and positioned over the glass-sensing window as indicated. B) The optical circuit board used in the device, based upon the reflectometric circuitry principles shown in Fig. 2.1.

However, a fundamental problem with this device (Fig. 2.2) was that hydrocarbon contamination of the soil samples often resulted in heterogeneous pocketing of the hydrocarbons across the interrogated surface. This problem was exacerbated by the presence of water. To overcome this problem, a hydrophobic membrane was used as an interface material between the sample and the glass window. Pilot experiments found the membrane readily absorbed liquid hydrocarbons from the sample. As this occurred, the membrane became translucent, resulting in a significant reduction in the amount of reflected light received by the phototransistor. With sufficient hydrocarbon contamination, the membrane became homogeneously translucent, which overcame the problem of hydrocarbon pocketing.

A third party manufacturer, Drallim Industries Ltd (Bexhill on Sea, UK) was commissioned to rehouse the optical circuitry into a 20" conical plastic housing. This prototype sensor (Fig. 2.3) was then adapted under licence by the UK National Grid Company, which has since merged with Transco to become National Grid Transco (NGT), for monitoring insulation oil leakage from sub-surface power cable networks (Ritchie *et al.*, (2000b). This is currently the only sub-surface monitoring system with NGT approval and a modified version is used specifically for installation in high voltage, self-contained, pressurised power cable systems (Drallim, 2002).

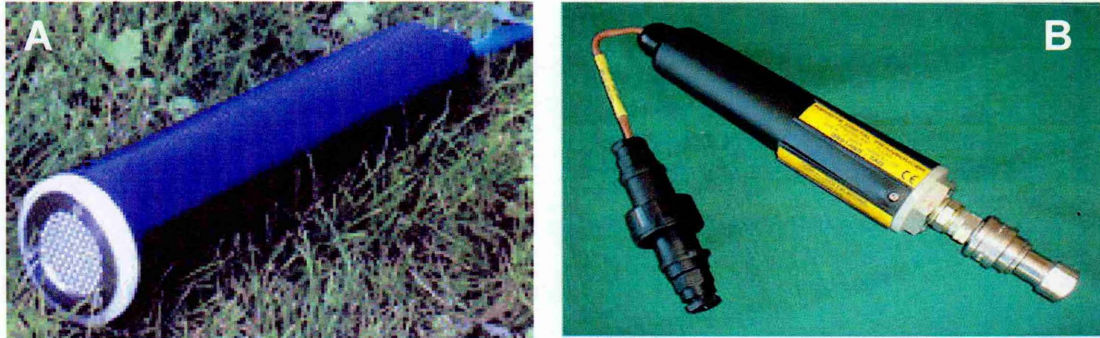


Fig. 2.3 Cable oil leak sensors

A) The prototype cable oil sensor developed by Cranfield University and manufactured by Drallim Industries Ltd; B) The cable oil sensor system employed by UK National Grid Transco. Both rely upon a hydrocarbon-sensitive membrane.

A similar sub-surface reflectometric sensing system, incorporating the same hydrocarbon-specific membrane was also reported using fibre-optic technology (Ritchie *et al.*, 2000c). The sensor circuitry incorporated a high-powered light-emitting diode (LED) and corresponding photodiode receiver mounted into a fibre-optic launch. However, in this system, multiple reflectometric circuits were housed within a separate microprocessor-controlled control unit. An additional signal amplification circuit was also required. Ten sub-surface fibre-optic heads were connected to the control unit via fibre optic cabling. These fibre-optic heads contained the hydrocarbon-sensitive membrane, which was separated from the fibre-optic tips using a 1 mm spacer and covered by an oxidised copper mesh. Each assembly was connected to a plastic housing containing the fibre optic bundles by industry standard SMA connectors (Fig. 2.4).

The preliminary experimental data reported by Ritchie *et al.*, (2000a, 2000c) for the two sensor systems (Fig. 2.2, 2.4) revealed several issues in relation to sensor design and performance, which clearly required further investigation and improvement. It should also be noted that the Drallim prototype sensor (Fig. 2.3) had not been evaluated by Cranfield, although it was later modified by the National Grid Transco Company, the modifications remain proprietary to NGT.

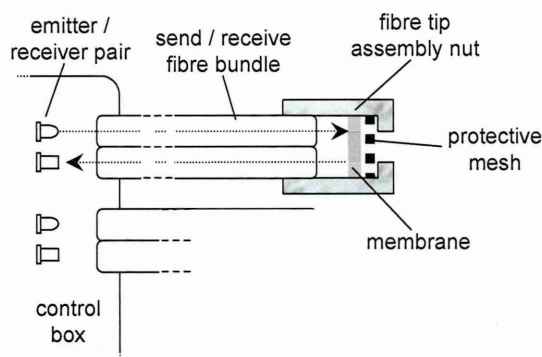


Fig. 2.4 Schematic diagram of the conceptual multi-channelled fibre-optic head.

The hydrocarbon-specific membrane (and protective mesh) was held to the fibre-optic bundle by standard SMA connectors. Generated and reflected light was relayed via fibre-optics back to a separate control unit containing the electronic and signal amplification circuits (Ritchie *et al.*, 2000c).

Briefly, the issues relating to the two early Cranfield sensor designs (Figs. 2.2, 2.4) and their performance were:

- Fuel concentrations (<10% w/w) could not be detected in dry soil.
- The reflectometric sensor response (using a 3 cm² membrane) to hydrocarbons was retarded when water was present (30% w/w) and did not respond when water content was 50% w/w or above in soil matrices.
- Different soil types/structure and their subsequent effects on sensor response had not been investigated.
- Alternative membranes had not been evaluated. Similarly, the ratio of fuel to membrane required to trigger the sensors had not been ascertained.
- Membrane reset (regeneration) was only observed under artificial drying conditions (50 °C) and had not been evaluated under natural environmental conditions. Similarly, reset times were greatly extended when water content was above 30% (w/w).
- The multi-channelled fibre-optic system required manual interrogation of the fibre-optics (by attachment of a PC) in order to obtain data from the fibre-optic heads.
- Not all the fibre-optic heads responded to hydrocarbons (250 ml) passed through a narrow, small-scale vertical pipe (10 cm dia., 46 cm length) filled with sand (1.83 g/cm³, 6.6% water).
- The order of sensor response varied and was not as predicted in logical sequence. Failure of response was attributed to air pockets. Migration of the fuel plume had not been addressed.
- An automated field-based data logging system was required for remote monitoring for either sensor system as a prerequisite for the conduction and evaluation of essential field trials.

Consequently, there remained no commercially available sub-surface sensor system that was capable of automated monitoring of hydrocarbon leaks *in situ*. This work addressed these issues and has resulted in the development of a new (patented) multi-channelled sub surface remote monitoring system which embodies the following improvements over it's predecessors:

- Novel hydrocarbon sensor
- Self-contained reflectometric sensor
- Compact, robust design
- On-board micro-printed circuit board (PCB) electronics
- Remote monitoring and data relay by way of integrated data logging unit
- Improved sensitivity to hydrocarbon fuel contamination
- Expandable multiple sensor capacity
- Cheap and simple to mass manufacture

This new composite, multi-channelled sensing technology, integrating aspects from the two former prototype systems was developed and evaluated during the course of this work. In order for this to be achieved, a novel real-time data acquisition programme 'PETROLog' was developed to evaluate sensor performance (2.6). In conjunction, a novel, automated data logging unit (DLU) was acquired for field trials (MedSci Consultants Ltd., Harpenden, UK) and subsequently developed throughout this work for remote monitoring with the new sub-surface fuel sensors (2.7).

2.3 DESIGN OF THE SUB-SURFACE FUEL LEAK SENSOR

The primary aim of this work was to develop a distributed network of fuel leak sensors capable of remote, long-term monitoring. It was desirable to reduce the overall length and diameter of the new Mk1 fuel sensors. A more compact sensor would be practically easier to install and distribute underground, especially if applied to existing UST sites via drilling. The prototype cable oil sensor (Fig. 2.3) was a relatively large, with a 12 cm diameter sensor head and 10 cm diameter sensing 'window' over which the fluoropolymer membrane was placed and covered by protective mesh. As a result of these dimensions, the matching sensor body (20 cm total length) housing the optical circuitry board and connecting cable had essentially 75% internal body cavity 'dead-space'.

In contrast to the previously reported multi-channel fibre-optic assembly, this sensor system would be redesigned and improved so that manual data collection (by an on-site operator) would not be necessary. The existing separate circuit control box and signal amplification

system was not desirable. Each sensor would be a stand-alone sensing unit. In case of any sensor failure, each one could be easily identified and replaced *in situ* without compromising overall monitoring.

2.3.1 Principle Mk 1 sensor design considerations

A number of new Mk 1 sensor designs were investigated, based upon the simple reflectometric principles previously described (2.1). Primary new design considerations were:

- Miniaturisation of the existing electronic circuitry
- Compact, robust sensor design
- Alternative body casing material(s)
- Suitability for multi-channelled, long-term monitoring
- Ease of manufacture
- Overall cost for marketing and economic purposes

In-house manufacturing was not feasible and so a local electronics manufacturer, Spectronic Devices Ltd., (Cranfield, UK) was consulted and commissioned to manufacture and supply the new Mk 1 sub-surface sensors.

2.4 MK 1 SUB-SURFACE FUEL SENSORS

2.4.1 Redesign of reflectometric electronic circuitry

A near infrared-emitting diode and an NPN silicon phototransistor pair, mounted together (on parallel axis) in a single, compact, plastic housing (Optek Technology Inc., Texas, USA) replaced the separate reflectometric circuitry previously employed by Ritchie *et al.*, (2000a, Fig. 2.2). The reflective unit was encapsulated within epoxy resin to reduce ambient light sensitivity, improving the signal to noise ratio and to seal the sensor at the cable exit point.

2.4.1.1 Principle of operation

The photodiode allowed a limited current flow in one direction (+ve to -ve) and emitted NIR light directed at the reflective membrane surface at a fixed distance (mm). The phototransistor sensed the reflected light at a base electrode and the light energy resulted in excitation of electrons within the NPN silicon component (Fig.2.5). This energy was passed to the collector electrode, which now converted the energy into current (I - power through the circuit) and passed (via an emitter electrode) on through the circuit. The current (I) conducted by the transistor was proportional to the incident light.

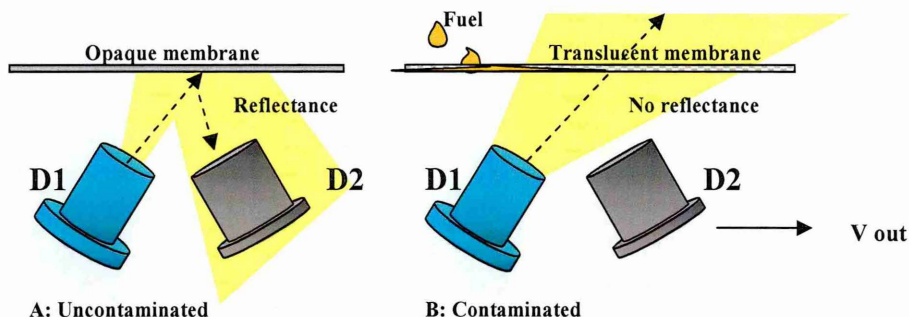


Fig. 2.5 Principle of reflectometric sensing incorporating a hydrocarbon-absorbing membrane for the new sub-surface fuel sensors. A) Under normal operating conditions (no hydrocarbons present), emitted light (D1) is reflected back from the membrane to the phototransistor (D2). The amount of light received by the phototransistor is monitored as a baseline voltage at a collector electrode. B) When the membrane comes into contact with hydrocarbon fluid, it turns translucent, allowing the majority of the light to be transmitted. The amount of reflected light therefore decreases, resulting in a change in the measured output voltage (V out).

By passing the voltage through a high resistor (10K Ω), the original voltage (5 V) was dropped to 0.4 V. When the phototransistor was operating in saturated light, the current (I) was produced from this collected energy. When light was lost (through a transparent membrane), a voltage change occurred which was detected by an on-board comparator, which provides the analogue output signal (V-out). This follows Ohm's Law, where V (voltage) = I (current) $\times$ R (resistance).

This adaptation then enabled complete miniaturisation of the internal electronic assembly which was built onto a micro-electronic printed circuit board (PCB, Fig. 2.6). In contrast to previous sensor designs (Ritchie *et al.*, 2000a, 2000b, 2000c), the electronic circuitry for the Mk 1 sub-surface fuel sensors was designed so that the signal (V-out) would *increase* with detection of hydrocarbon fuels. The analogue output signal gave a low resting signal (~ 0.4 V) when the membrane was uncontaminated, opaque and highly-reflective. Increased membrane translucency in response to fuel contamination (and subsequent loss of reflected light/collected current) would *increase* the signal output (V) to a maximum of 5V. Therefore, the signal would be hypothetically proportional to the degree of contamination.

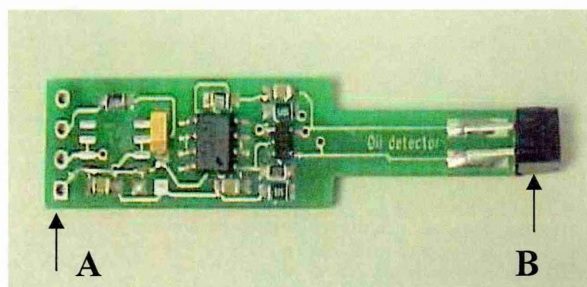


Fig. 2.6 The miniaturised Mk 1 reflectometric PCB

A) The 4-wire connectors for the connecting cable are indicated (5 V power supply, ground, V-out signal, digital signal); B) The combined NIR emitter/receiver pair.

2.4.2 Redesign of the reflectometric sensor body

Electronic miniaturisation meant that the entire sensor body could be now reduced in size, minimising any ‘dead-space’ and improve the sensor design overall. It was proposed that a smaller sensor would be easier to manipulate for sub-surface monitoring and show greater sensitivity in response to free-phase hydrocarbons as a result of the smaller, membrane surface housed within the sensor head. Tubular brass was selected for the sensor casing based on the following criteria:

- Brass components were easy to manufacture due to the softness of the metal
- The sensor casing was highly robust therefore suitable for long-term, sub-surface use
- Brass was known to be highly resistant to oil, fuel, water and rust
- Brass was an aesthetically attractive material.

The new Mk 1 sub-surface fuel sensors (100 mm length, 16 mm dia., Fig. 2.7), consisted of two primary parts:

The sensor body. This housed the PCB circuitry and the connected NIR element, which was situated directly behind the glass sensing window (11 mm dia.) and also the internal cable connections. The entire sensor body was fully sealed with epoxy resin to prevent internal water/fuel contamination.

A separate sensor head assembly. This housed a protective 1 mm thick anodised copper mesh at the front of the sensor head. The diameter of the hydrocarbon-specific membrane was correspondingly reduced to 11 mm (dia.). This was placed directly behind the mesh. A 1 mm oil-resistant O-ring was placed onto the glass window (of the sensor body) and the head assembly then screwed in place. The O-ring ensured a fixed distance (2 mm) from the NIR sensing mechanism and also gave a watertight seal.

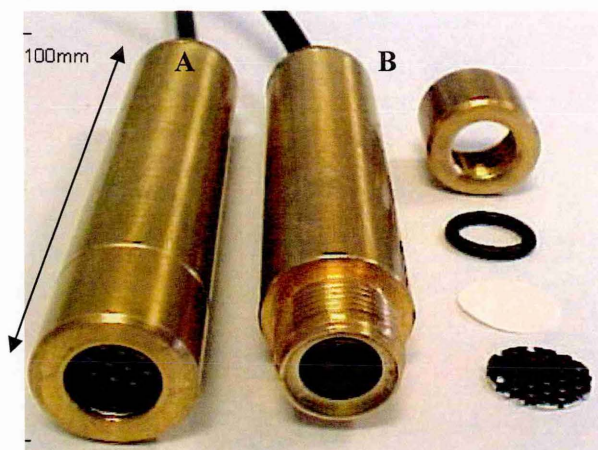


Fig. 2.7 Mk 1 sensor sub-surface fuel sensor

A) The fully assembled Mk 1 sub-surface fuel sensor. B) Exploded view of the sensor head assembly. The O-ring spacer, membrane and protective mesh are shown as placed into the sensor head.

2.4.3 Choice of hydrocarbon-specific membrane

A broad range of commercially available hydrophobic membranes were evaluated by laboratory-controlled exposure to a variety of hydrocarbon fuels (4.2.3). Polyvinylidene difluoride (PVDF) membrane (0.2 μ m pore size - 'Fluorotrans' (Pall-Gelman Ltd., Portsmouth, UK) was found to be the most effective material. Naturally hydrophobic, the membrane was highly reflective in its opaque state, providing a low sensor background signal. Maximum translucency with minimal fuel (8 μ l) was achieved, which in turn enabled a maximum sensor signal of ~ 4.6 V. Extensive evaluations found that this membrane showed the most rapid and complete regeneration after repeated exposure to fuels (4.2.3). Further selectivity experiments revealed that this membrane was also highly resistant to biofouling (4.2.6) and suitably robust for long-term field use (4.3.2).

2.5 CHRONOLOGICAL DEVELOPMENT OF THE FUEL LEAK SENSOR

The redesigned Mk 1 fuel leak sensors were extensively evaluated and improved throughout this work (Mk 1 to Mk 4), the ultimate aim being commercialisation of a multi-channelled distributed sensor system for long-term, *in-situ* sub-surface monitoring. Whilst these evaluations are reported in full under individual experimental sections (Chapters 3 & 4), Table 2.1 summarises the whole chronological process, which ultimately led to development of the superior Mk 4 sensor. Relevant experimental results have been cross-referenced for convenience.

2.5.1 Preliminary field trial evaluation of the Mk 1 fuel leak sensor

A preliminary outdoor monitoring experiment with two Mk 1 sensors (4.3.1) had proved unsuccessful due to: A) excessive water saturation of the sand within the holding tank as a result of inclement weather, B) clogging of sensor heads by sand. A number of design issues were rapidly identified. Based on these issues, modifications were applied to the Mk 1 sensor design – the result was the Mk 2 sensor.

- Each Mk 1 was found to draw ~60 mA current, which rapidly depleted the data logging unit's (DLU) on-board power supply (8V Ni-Cad). The electronic circuitry was modified in the Mk 2 sub-surface fuel sensor to reduce the current demand to ~10 mA per sensor.
- The on-board comparator was removed, as this was believed to be the cause of the excessive power demand. The signal fluctuations were most probably due to environmental temperature fluctuations.
- Each Mk 2 sensor was to be supplied with 15 m insulated connection cable to enable positioning of the sensors over larger areas. These were to be supplied with simple, RS232 serial interface sub-D connectors.
- The prototype DLU unit required manual bare-wiring of each sensor, which could easily have short-circuited if this was performed during monitoring. A simple, sensor interface board was built (2.7) for rapid and easy connection or removal of individual sensors from the DLU during field monitoring trials, if required.

2.5.2 Mk 2 sensors optimisations

Extensive characterisation and evaluation of the Mk 2 sensor response to active phase fuel movement through soil (4.2.4) and its response to fuels in water were performed under controlled laboratory conditions (4.2.1-4.2.2). These evaluations prompted the following modifications, which constitute the Mk 3 design.

- The O-ring spacer was found to impede membrane contact with hydrocarbon fuels through siting the membrane further back in the head casing and so this was removed.
- The protective mesh also impeded the transport of fuel to the membrane surface and so this was discarded.

- The sensor head casings were then re-machined to reduce the front recess (dead volume) at the sensor face by 5 mm. This effectively brought the membrane closer to the soil interface, minimising potential clogging and improving absorption of any hydrocarbon fuels present.
- As the membrane now lay directly over the glass-sensing window (housing the NIR photodiode/transmitter pair within the sensor body) and was held in place by the sensor head, improved baseline signals were achieved. The distance of the NIR lens from the reflective membrane surface directly influenced the overall performance of the NIR pair. By reducing the spacing (by 2 mm) between the NIR lens and the reflective membrane, resolution was improved and the amount of reflected light received at the transmitter's base electrode increased, lowering the baseline to ~0.1 V, increasing the dynamic range of the Mk 3 sensors.

2.5.3 Remote monitoring with the Mk 3 sensor

The modified Mk 3 sensors were extensively evaluated, under conditions designed to mimic the natural environment, in long-term field trials (4.3.2). After repeated usage and exposure to contaminants, a number of issues were raised:

- The glass disc covering the sensing window deteriorated around the edges. The sensitive electronic circuitry housed behind was then vulnerable to exposure to water/fuel. Increased sensitivity to temperature changes would also be expected. The reasons for the deterioration were most likely to have been through repeated tightening of the sensor head assembly. The Mk 3 design did not allow for replacement of the glass sensing window.
- The cable exit seal were also found to deteriorate after repeated exposure to fuels (4.4). This was exacerbated by the fact that the sensors were removed from the soil by pulling on the cables, this usually required significant force. Compacted soil (within the lysimeter) could not be excavated to remove faulty sensors during active monitoring.
- A number of sensors (3) malfunctioned during the course of this work. Due to the Mk 1-Mk 3 design of the sensor body being completely sealed with epoxy resin, examination of the electronics to determine the cause of the fault was not possible.

2.5.4 Final optimisations and redesign of the Mk 4 sub-surface sensors

The issues raised by the Mk 3 sensor evaluations (2.5.3) were fully addressed and resolved in the final design of the superior Mk 4 sensors (Fig. 2.8), which were then manufactured by a local engineering company (BELA Electronic Designs Ltd., Kempston, UK).

- Access to the internal components was enabled, together with a reinforced cable exit seal for increased strength.
- The sensor body cavity was not sealed in order to allow access to the electronic circuitry, which was itself sealed with a removable heat-shrink plastic for insulation.
- The sensor head was re-bored and incorporated an embossed front brass rim to protect the glass-sensing window edge.
- Reducing the length of the connections to the NIR pair, improved overall board strength and shortened the PCB board.

Fig. 2.8 and 2.9 illustrates and visually compounds the chronological development of the Mk 1 sub-surface fuel sensor to the superior Mk 4 design.

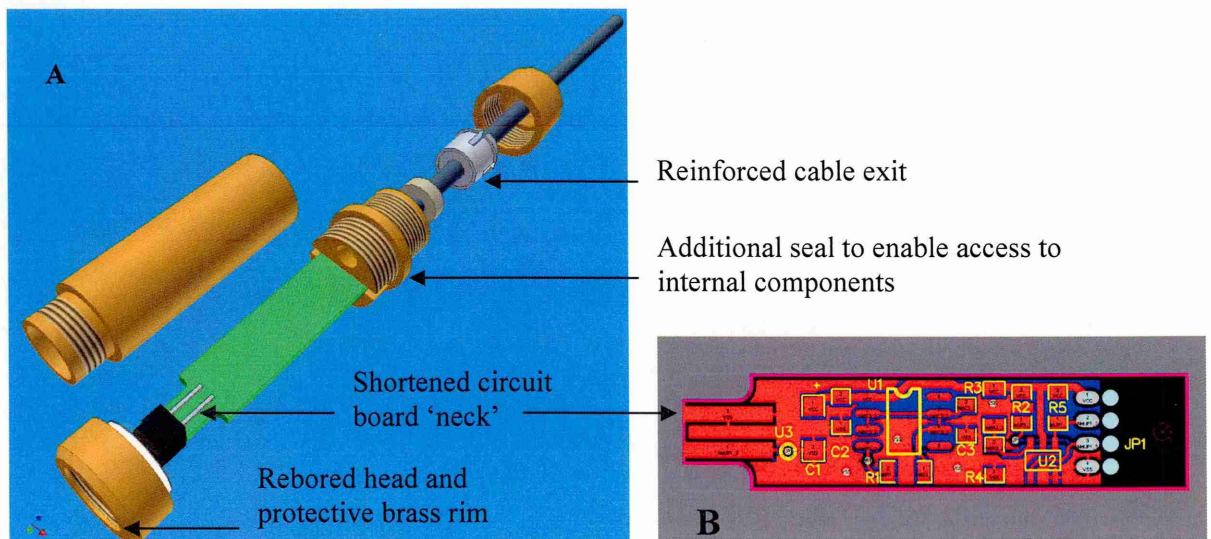


Fig. 2.8 Mk 4 sensor design

The computer-aided design of the Mk 4 sensor, as used for manufacturing, is shown. A) Sensor body, showing the reinforced cable exit and open-access design; B) redesigned electronic PCB with the reduced 'neck' connecting the NIR emitter/detector pair.



Fig. 2.9 The development of the Mk1 sub-surface fuel sensor to the superior Mk 4 design. A) Assembled sensors Mk 1-4 (left to right) showing decreasing head recesses; B) Mk 1-4 sensors showing sequential reduction of head length and minimised front recesses. The protective glass lip on the Mk 4 sensor is evident; C) Mk 4 sensor (rear); D) Mk 4 sensor with head assembled (face front); E) Sealed internal components of the Mk 4 sensor and reinforced cable exit assembly. The NIR unit is shown top. The reinforced cable exit (bottom) is shown dismantled.

2.6 DEVELOPMENT OF DATA ACQUISITION TECHNOLOGIES FOR INCORPORATION WITH THE SUB-SURFACE FUEL SENSORS

2.6.1 Design & development of a laboratory-based data acquisition system

There was no existing software to monitor the Mk 1 sensors for laboratory-based evaluations (4.2). Consequently, vital preliminary work was to construct an appropriate data acquisition (DAC) interface that could operate to connect the sensors to a PC via a software programme. The electronic interface and DAC is described in 2.6.1.1. The specific software-monitoring programme that could operate within a Microsoft Windows environment was developed as part of this work and is described in Section 2.6.1.2.

2.6.1.1 Electronic Interface

For the laboratory-based evaluations, sensors were powered using a 5 V laboratory power supply unit (PSU). In order to monitor the sensor signals, it was necessary to design an interface system to connect the sensors to a standard PC (Fig. 2.10).

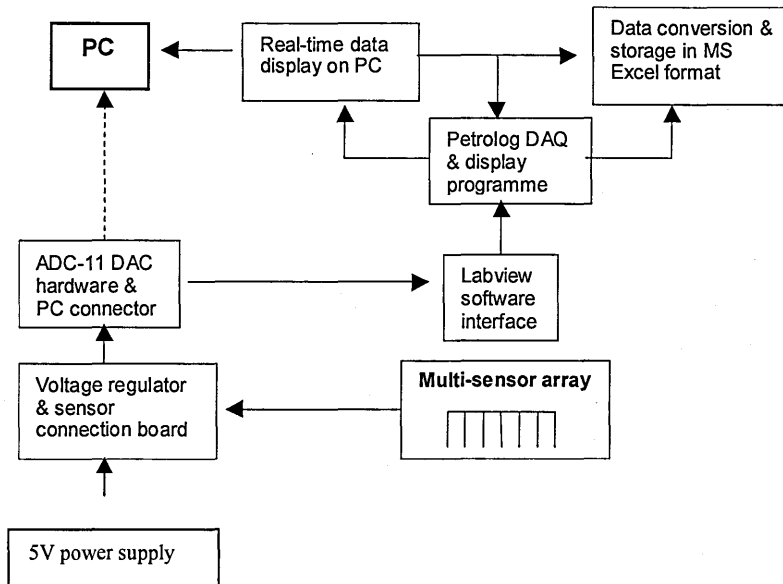


Fig. 2.10 Schematic flow chart showing required interfaces for lab-based sensor data acquisition. Multiple sensors were connected via Sub-D connectors routed through the voltage regulator board to the ADC-11 DAC. The novel PETROLog programme enabled multiple parameter data acquisition, real-time on-screen display of sensor signals and conversion into spreadsheet (MS Excel) format for post-experimental analysis.

A commercial multi-channel data acquisition card (ADC-11), supplied by Pico Technology Ltd, (St Neots, UK) was used to monitor the sub-surface fuel sensor's signal output voltages using a standard PC. The ADC-11 unit provided 11 channels of analogue input and connected to a

standard computer via the supplied 25-pin PC parallel port connector. However, the ADC-11 had an input voltage range of 0-2.5 V, whereas the sensors were designed to output >5 V. Therefore, an interface board was designed and manufactured in-house in order to channel each sensors' signal output on a separate circuit through a potential divider (2 x 100 K resistors). The maximum signal output voltage for each sensor was reduced to a maximum of 2.5 V, without any loss of sensitivity.

2.6.1.2 Development of the data acquisition & real-time display programme

For simultaneous, real-time monitoring for the sub-surface Mk1 sensors, a novel, in-house data acquisition software programme 'PETROLog.exe' was designed and written using 'Labview'. 'Labview' is a graphical programming language, also referred to as G-language, developed by National Instruments Corp. (Newbury, UK). The G-language enables programmers to develop applications, with or without data acquisition, in terms of visual icons and wiring instead of text-based syntax, as is the case with conventional programming (C++ or Pascal). Conventional programming uses 3 layers of code - high-level code, or source code; mid-level code and low-level machine code. Labview uses two code levels. High level code represents the virtual instrumentation (vi) file the programmer creates, a 'vi' is defined as the basic file unit in Labview. The low-level code is what Labview automatically compiles the 'vi' into and not visible to the programmer or the end user. A schematic diagram summarising the programme structure is shown in Fig. 2.11).

2.6.1.2.1 User interface screen

The first step in the programme development was the design of the user interface screen (Fig. 2.12). To enable real-time display of each sensor signal, acquired from the ADC-11 unit, the multi-channel data was displayed within a standard 'waveform chart'. The chart was programmed to display data in Strip mode. This provided continuous real-time data display as the data was collected. The chart screen auto-scrolled as the data was received and displayed. Current data was therefore always visible and old data values were stored in the PC's virtual memory, rather than being over-written. The y-axis was set to represent the sensors' optimal signal range of 0-2.5 V. The x-axis was default categories, which logged the number of collected data points. Each number could represent the variable times of data acquisition, for example, 1 s or >10 minutes between sampling, as determined by the programmer/user.

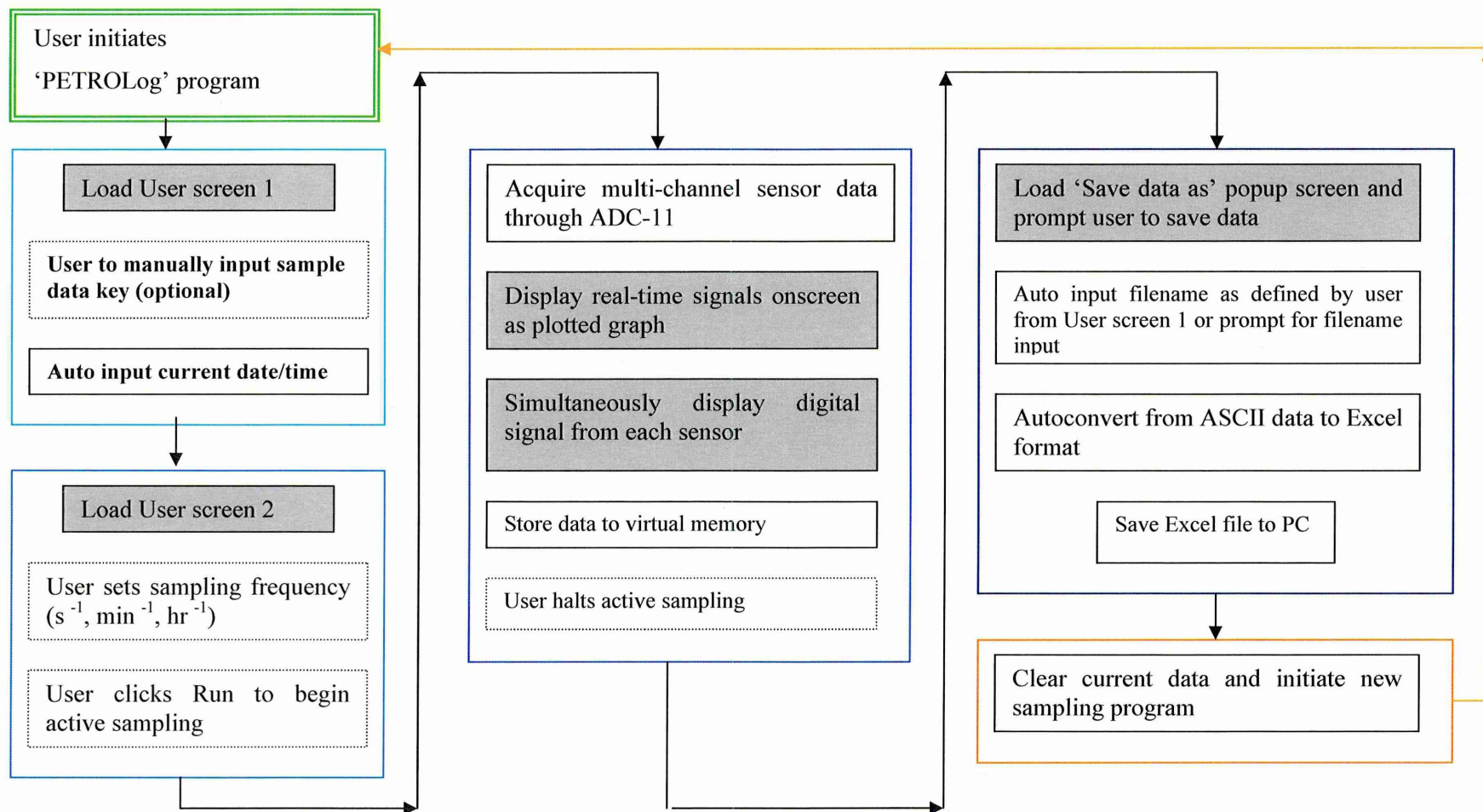
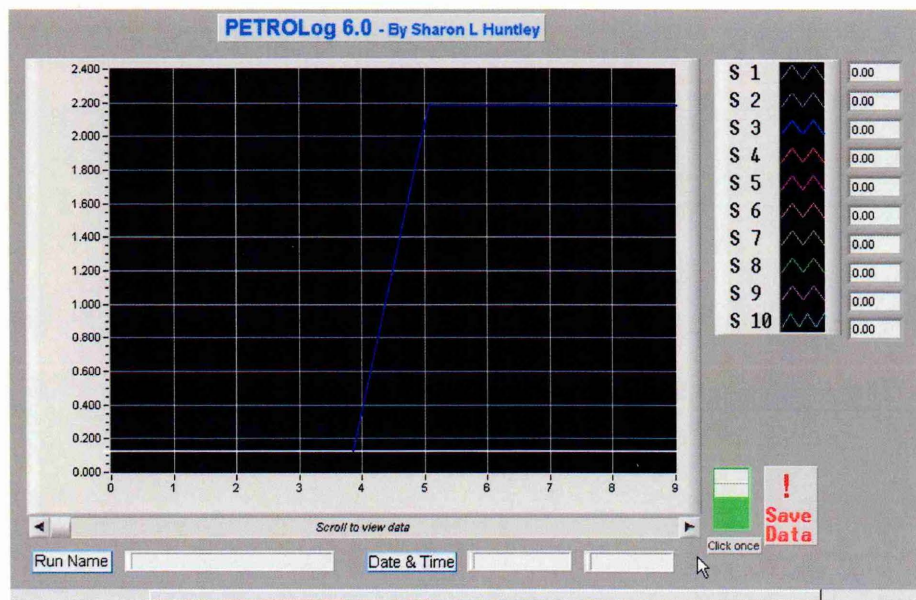


Fig. 2.11 Schematic plan for design and use of the novel PETROLog data acquisition programme, written in Labview.

There were five Labview sub-programs used within the PETROLog data acquisition programme. User interface screens (on-screen displays) are shown in grey boxes. User input is indicated by dashed line boxes. All other processes were programmed to automatically activate.

The 'Run/Stop' Boolean control (Fig. 2.12) related to a True or False value within the hidden programming. By default, this was programmed in the Run position at the start of each experiment. When manually stopped by the user, a pop-up screen would appear to prompt the user to save the data.



Real time monitoring example

The X-axis logged data as a timed sampling index (hours: minutes: seconds). If sampling was per minute, the sampling time shown should be converted as follows: e.g. 2:26 should be interpreted as $(2 \times 60) + 26 = 146$ minutes.

Fig. 2.12 The PETROLog.vi user interface screen (in blue) within the Labview default window. The real-time multi-channelled sensor data is displayed within the chart window. A triggered sensor response (sensor 3) is shown. Untriggered Mk 3 sensors all had baseline signals of ~ 0.1 V, shown at the bottom of the chart. The corresponding signals (in decimal format) from the last corresponding data plot shown on the chart are displayed next to the sensor legend to the right of the screen, when the program is active. The monitoring file name (shown at the bottom of the interface screen) is inputted by the user in a pop-up window (sub-programme) prior to running the programme (Fig. 2.11) and is displayed (with auto-input date/time) throughout active monitoring.

2.6.1.2.2 Data acquisition programming in Labview

The PETROLog (vi) programme was developed within two frames or programme cycles. The first programme cycle (Fig. 2.13) commenced at the start of the programme. When the end user clicks on the default Run arrow (Fig. 2.12), a pop-up screen prompts the user to input sampling details within a text box 'Run Name'. Additionally, the Get Date/Time string converted a time-zone-independent number to a date and time string in the configured time zone for the users' computer. This automatically input the correct date and time and correspondingly displayed this information on the main user screen.

The Get Operator Info.vi (palette menu) was a sub-vi incorporated into the first frame of the programme. This took the three strings of data inputted by the operator in the name, date, and time text boxes when the vi first ran. Each input string was then sent into a 3-channel array.

Once confirmed as correct by the user, each element of the array was then concatenated into a single concatenated string of ASCII data.

This data then passed into another sub-vi, 'Write Characters To File', which saved the information into a user-defined data file. Once this was imported into a Third-Party spreadsheet application (Microsoft Excel), the three data strings would be placed into the first cell (A1) to record the experimental information. After data acquisition, the recorded data for each sensor would then be added to the file and the user would be prompted to resave the file containing all the information.

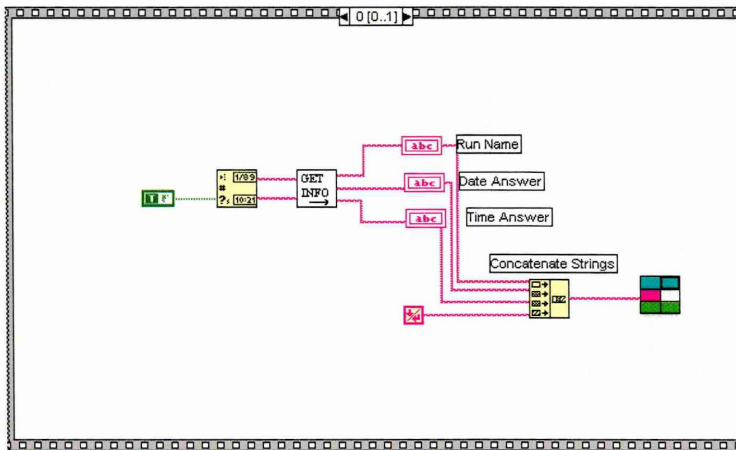


Fig. 2.13 First level programming (frame 0)

Once the run button has been activated, the programme runs through this programme cycle. A pop-up screen appears (Fig. 2.11) asking the user to input the experimental run name and conform the date and time. This data is then stored and appears on the main user screen.

The PETROLog.vi then passed to the second frame (layer) of programming incorporating the main data acquisition programming (Fig. 2.14). This enabled the signal readings from each sensor to be displayed on screen and simultaneously logged continuously into the named previously created file. The Port control instructed the vi to take the signal data received from the ADC-11 card connected to the computer's parallel port. For each sensor, a sub-vi ADC-11.vi created specifically for the ADC-11 (downloaded from www.PicoTech.com) was connected to the Port control.

Each set of data (from each sensor) was then fed into a Build Array. The function of the Build Array this was to append any number of array or element inputs in top-to-bottom order to create array with appended element. Initially, this function had two element inputs (Fig. 2.14). This could be expanded to build an array of n -dimensions, although each array input needed to be of the same dimension, n , and each element input required $n-1$ dimensions. As the data acquisition consisted of multi-channelled data that was to be converted into columns of data, a 2D array

was built by connecting 1D arrays to element inputs and 2D arrays to the array inputs. Output data from the array was then split into two paths. The first passed into another array, which separated the data sets and transferred them to the waveform chart, which then displayed the signal data for all the attached sensors. The second channel passed through the While loop into a Sub-vi 'Write to Spreadsheet File.vi' in order to save the data. These paths were controlled by the Run/Stop control on the user interface screen.

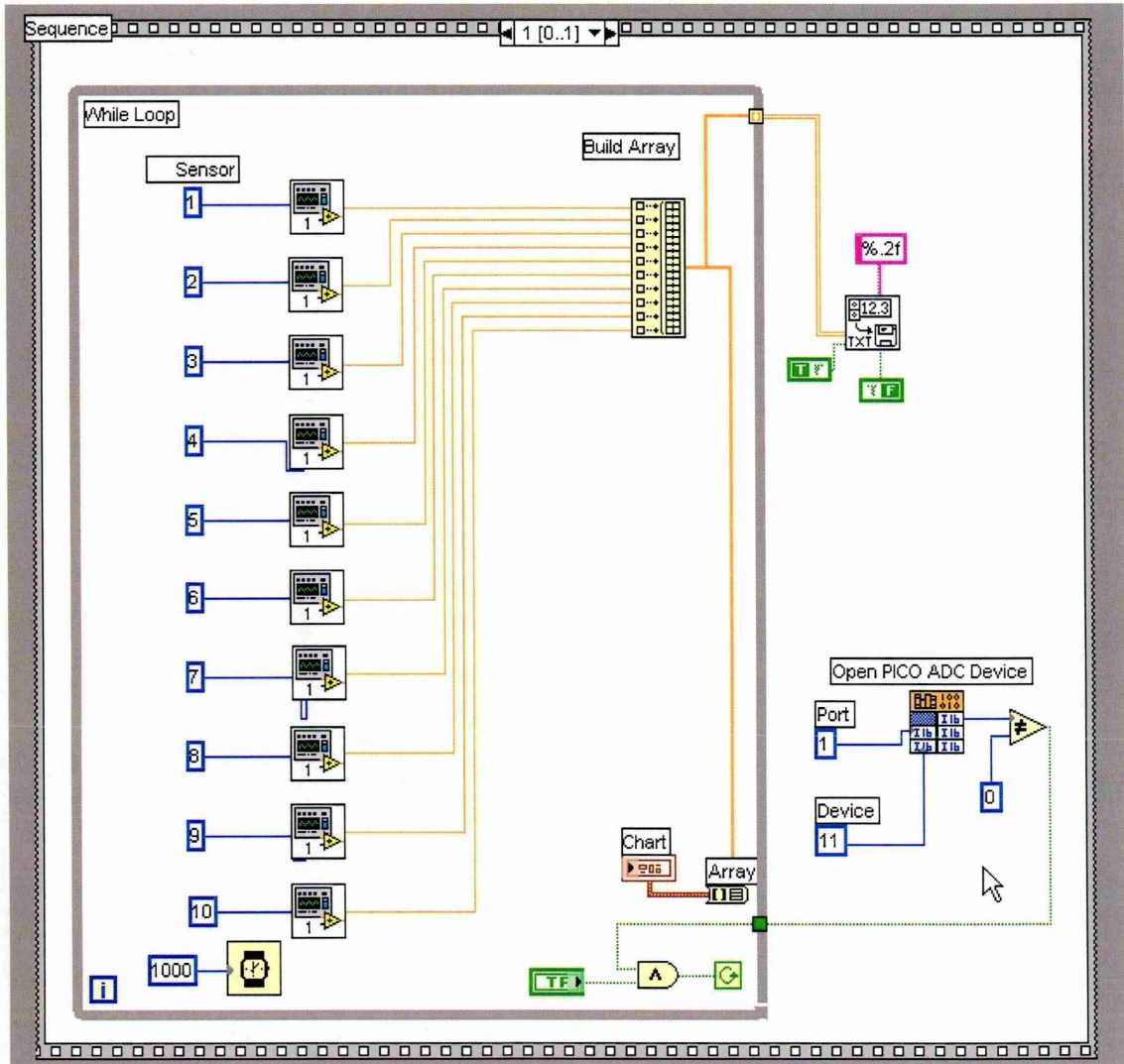


Fig. 2.14 2nd level programming (frame 1)

The programming within this frame controls the signal acquisition from the ADC-11 and displays the signals for each sensor on-screen (programming for 10 sensors is shown). All data is saved within the memory and then converted and saved into a spreadsheet.

The ‘Run/Stop’ shown on the user panel (Fig. 2.12) was a Boolean control command and related to a True or False value within the programming. By default, this was set via the mechanical action settings to ‘Latch When Released’. The vi was therefore automatically set to run. The Stop command would stop the vi and end data acquisition. At this point, a sub-screen would appear prompting the user to save the data. The Run switch would then reset back to the Run position on screen. Within the programming screen, this correspondingly reset the ‘While

Loop' to the True position, after generating False when the user had activated the Stop button on screen. At this point, the second data stream passed into the 'Write to Spreadsheet ' pathway.

The Write to Spreadsheet File.vi converted the 2D array of single-precision (SGL) numbers to a text string and wrote the string to an existing byte stream file. This vi opened the file before writing to it and created a text file readable by most spreadsheet applications by calling the Array to Spreadsheet String function to convert the data. The Write to Spreadsheet.vi then prompted the user to save the amended file and close the file.

Once saved, the American Standard Code for Information Interchange (ASCII) format data was imported into a standard spreadsheet (Microsoft Excel) and converted from ASCII text to a standard MS Excel workbook format. Using the Chart function, the data was displayed graphically to replicate the real-time data acquired post- experimental signal sampling.

Summary: Data acquisition programming

- Once the PETROLog.vi programme had been developed, it could be opened using Labview. The front panel of the on-screen display represented the user interface. The programming wires and icons were hidden behind this interface. These could be viewed from the diagram window by the programmer but could be hidden from end-users by assigning a password.
- The developed programme enabled flexible monitoring of an array of sensors and real-time display on a standard PC was achieved. In conjunction, all data was simultaneously saved to a spreadsheet. The latter enabled post-sampling analysis of the sensors' response times and signals.
- The programme had the flexibility to alter sampling rates, for example, per second, minute, hour, day etc., and to increase or decrease the number of connected sensors. Due to the limitations of the ADC-11 used, this was restricted to a maximum of 10 sensors.

2.6.2 Field-based data acquisition & transmission

A novel, multiple parameter data acquisition unit, with a re-programmable micro-controller (Microchip™), integrated with a GSM-based unit (Global System for Mobile communications), was provided by MedSci Consultants Ltd (Harpندن, UK) and used for all field-based sensor trials (Fig. 2.15). The MedSci data logging unit (DLU) was specifically adapted throughout this project (in conjunction with MedSci) to operate with the multiple sub-surface fuel sensors for

remote field monitoring purposes. Technical specifications of the data-logging unit (by category) are provided in Appendix 1.

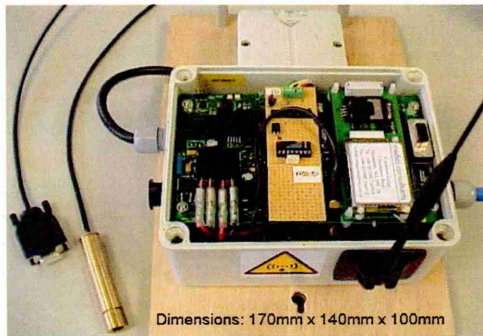


Fig. 2.15 Field monitoring MedSci DLU

Data logging unit (MedSci Consultants Ltd) and a Mk 2 sub-surface fuel sensor. The sensors were originally bare-wire connected through the external wiring input box (shown top centre). The adapted unit incorporated 7 external 9-pin sub-D type connectors for connecting sensors, replacing the original wiring box. A later Mk 2 sensor with sub-D connector is shown for size.

2.6.2.1 Principles of data acquisition

The sensors were connected to the unit via an external wiring box and both the unit and sensors were powered via an on-board 8 V Ni-Cad battery. The unit was programmed using a novel, specific Windows software program 'OilProbe.exe' to enter the required data acquisition profile, e.g., sampling intervals, monitoring station name, recipient e-mail address(es). This set-up information was then downloaded directly from a PC (or laptop) to the unit via a 16-pin serial cable. Once programmed, the unit then operated independently and activated the sensors at pre-defined sampling intervals.

Each sensor's analogue output voltage was sequentially connected at the multiplexing stage to the units' 11-bit analogue to digital converter (ADC-11). The analogue value was converted into binary code and combined with the current time from the real-time, on-board system clock. These codes were then stored in the unit's random access memory (2 Kb RAM) along with the date and time of sampling. This sequence repeated at the pre-set intervals defined by the user. Between these sampling events, the entire system went into a power saving mode to prolong battery life.

2.6.2.2 Principles of data transmission

Through the integrated GSM-based mobile communications unit, data could be delivered periodically to the user via Internet communications and digital telephone networks using Short Messaging Service (SMS) notifications via an on-board modem. At intervals set by the user, the unit made a cellular telephone call and connected to the Internet using a host Internet service

(Microsoft Outlook). Once a connection was established, the stored data (in the unit's system RAM) was encoded as a binary data file attachment (*.dat file). Using standard Internet protocols, an email was sent to one or more host email addresses (data recipient) defined by the user during programming. The unit then confirmed correct transmission, logged off the host server and disconnected the call. Sampling of the sensors then resumed and continued until the sampling and/or unit stop time set by the user.

The sample data was received by the email recipient as an attached binary data file. Using a simple executable software programme, 'ilogExtract.exe', the binary data was extracted and converted into a comma-separated variable (*.csv) file format. This could then be imported and saved within a spreadsheet programme (Microsoft Excel) for analysis.

2.7 CHRONOLOGICAL DEVELOPMENT AND EXPANSION OF THE MEDSCI DLU IN CONJUNCTION WITH THE MULTIPLE SUB-SURFACE FUEL SENSORS

As previously stated, the DLU was specifically adapted (in conjunction with MedSci) as a direct result of field monitoring trials with the multiple sensors. This chapter provides a chronological sequence of events and subsequent improvements made to the DLU/sensor system throughout this project.

- The DLU originally only had the capacity to monitor 3 sensors. This was returned to MedSci in order to increase the system's RAM memory. The unit was reprogrammed to monitor 8 channels. The eighth channel was reserved for monitoring battery power. With the new configuration, the unit could operate for an estimated 6 months.
- The original external sensor connection box was removed. Using a standard electronic board, the DLU power supply was channelled through the board to create an active circuit. Seven male Sub-D connectors were fixed to the board and wired back to the on-board multiplexing assembly. This relayed the analogue signal from each connected sensor (via the sub-D connectors) back to the DLU. The new sensor interface junction box was housed within a waterproof plastic box and sited directly underneath the DLU (Fig. 2.16). This enabled simple *in situ* connection (and disconnection) for 7 sensors.

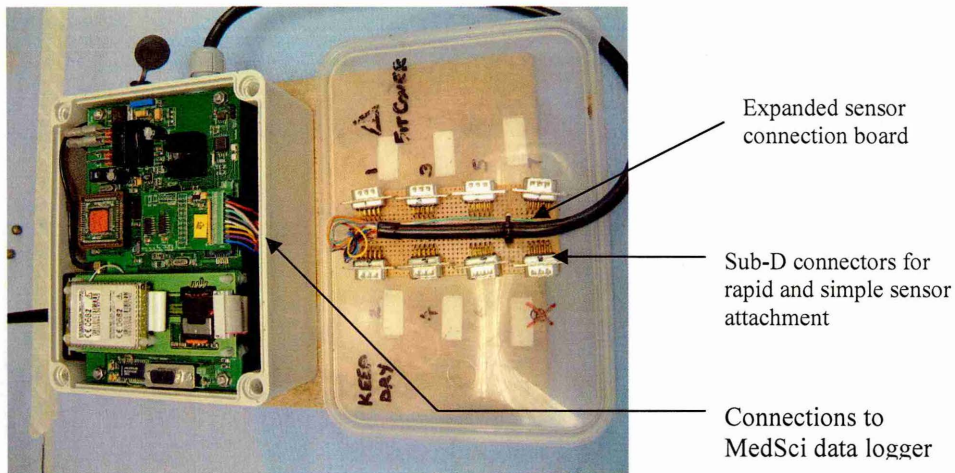


Fig. 2.16 Fuel leak field monitoring system with expanded sensor connection board.

The adapted DLU unit and sensor connection board enabled 7 sensors to be easily connected in-situ via the incorporated external 9-pin sub-D type connectors. This replaced the original wire connection box shown in Fig. 2.15.

2.7.1 Software updates & user features

- The DLU software was upgraded to enable separate parameters to be set for each sensor. This would enable different sampling frequencies for each individual sensor (minutes, hours, days), as required. Alternative sensors could be connected for enhanced monitoring, for example, a temperature or pH sensor. Additionally, if the full array of sensors were not required, only the desired number could be activated, which conserved the unit's battery power.
- An additional programming feature was included to allow channel 1 set-up information to be copied to all the remaining channels. This removed the need to set individual sampling parameters (start and end times, rate of sampling) for each sensor.
- The unit was designed originally to function with Windows 98 operating system (OS). It was found that using an alternative OS, such as Windows NT or Windows 2000, the unit would not program correctly. As a result, it remained active (awaiting programming) and so rapidly depleted the battery supply (within 24 hr). The software was subsequently upgraded to function with more modern operating systems.
- A modified 'sleep mode' programme alteration was also made. This would automatically shut the unit down if no programming download information had been received after a 30 min period (this prevented battery depletion if the unit was left active without being

programmed). Battery fluctuations had been shown to increase as the battery depleted and caused a corresponding fluctuation in sensor signal (4.3.2.2).

- For on-site pre-monitoring diagnostic checks, a new test mode was incorporated which allowed the unit to be instructed to send an email immediately on activation (this allowed the unit to be tested on-site overriding the internal email interval set-up). Diagnostic output was also converted to text, rather than in the previous code, which allowed test data outputs to be easily understood by a basic trained operator.

2.7.1.1 Internet access whilst monitoring

Access to acquired sensor data via the Internet, whilst still monitoring *in situ*, was also developed. The telemetry system applied a new approach to delivering data from remote locations whilst retaining the benefits of established technologies. By integrating the capability of wireless and Internet connectivity within the same unit then communicating the data directly to a central database engine, data could be accessed via the Internet.

The user simply logs to the central control site (<http://www.teleonix.com>) using a private user name and password. The most recent signal from each sensor is displayed on screen (Fig. 2.17). Users can also select a time window to view all data (for a single sensor) for the specified timeline period at once.

This concluded the development work for the sub-surface monitoring system. The experimental methods and results, which enabled the development of the sub-surface monitoring system with the final Mk 4 sensor design are reported in Chapters 3 and 4. Chapter 4 concludes with an overall, general discussion (in relation to sensor performance and monitoring trials) and suggests other potential applications for the system.

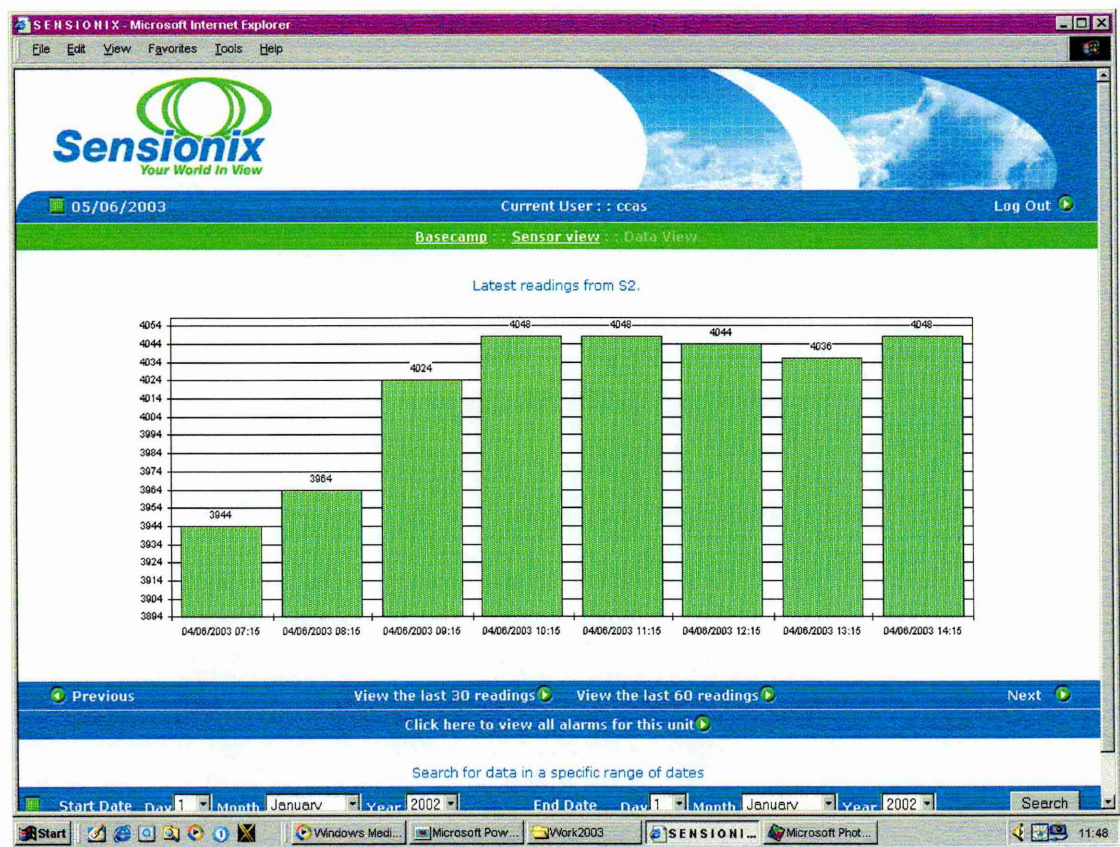


Fig. 2.17 On-line screen showing hourly sensor data

The most recent lysimeter monitoring for sensor 2 is shown. The last 8-hourly readings had been selected, but this option could have been for the last 30 or 60 hours (shown at the bottom of the screen). Sensor signals are shown on the y-axis, with the recorded date & time of sampling on the x-axis. This data was viewed off-site through a remote Internet log-in procedure.

CHAPTER 3

MATERIALS & METHODS (Sub-surface monitoring)

This chapter details the materials and methods used for the experimental work for the development of the sub-surface sensors and data acquisition technologies. The experimental results are reported in Chapter 4, which in turn directly contributed to the chronological development of the sub-surface monitoring system reported in Chapter 2.

3.1 GENERAL REAGENTS

The following fuels were used for all sensor evaluations: Standard grade unleaded petrol (95 octane rating) was obtained from a local Texaco service station; standard agricultural grade diesel (Texaco 35 second (sec) gas oil, supplied by Cranfield University, Silsoe, UK); laboratory grade MTBE (Sigma-Aldrich, Gillingham, UK); central heating oil (Texaco domestic 28 sec kerosene, supplied by Cranfield Oils, Cranfield, UK).

Deionised RO water was prepared in-house by ion exchange and reverse osmosis (Elgastat Group, High Wycombe, Bucks, UK). This was used for all fuel mixtures, unless otherwise stated. Fluorotrans membrane (Pall Europe Ltd., Portsmouth, UK) was used for all evaluations with the sub-surface sensors, with the exception of the work described in 3.2.3 (alternative membrane evaluations). After all experiments, sensors were thoroughly washed in detergent, dried and cleaned with Analar-grade methanol (MeOH, Sigma-Aldrich, Poole, Dorset, UK).

3.2 LABORATORY-BASED EVALUATIONS

3.2.1 Laboratory-based data acquisition

For all laboratory-based evaluations, Mk 1-4 sensors were powered by a standard laboratory 5 V power supply and routed via a voltage interface board to an ADC-11 multi-channel data acquisition card. Sensor data was acquired using a novel, in-house designed data acquisition and PC software programme PETROLog (2.6.1.2). All experiments were carried out under controlled conditions at RT (22 °C).

3.2.2 Evaluation of sensor sensitivity to fuels in water

3.2.2.1 Detection of sheared fuels in water (sensors sub-surface)

Dilutions and sampling were performed in 5 ml low-density polyethylene (LDPE) capped sample vials (Merck BDH, Leicester, UK). A range of fuel/water mixtures were prepared in 2 ml volumes. A magnetic PTFE cylindrical stirring bar (12 x 4.5 mm) was used to shear the fuel/water mixture (Fig. 3.1) by rapid stirring (maximum 1000 rpm) on a Hanna HI-190M

magnetic stirrer (Hanna Instruments, Leighton Buzzard, UK). Samples were mixed for 10 min prior to evaluation. This was continued during sampling. The PETROLog software was programmed for 1 s sampling. Baseline signals were established for 5 s. Sensors were then submerged ~ 1 cm below the fuel/water surface (Fig. 3.1). Data was recorded for 30 s. Tests were performed in duplicate.

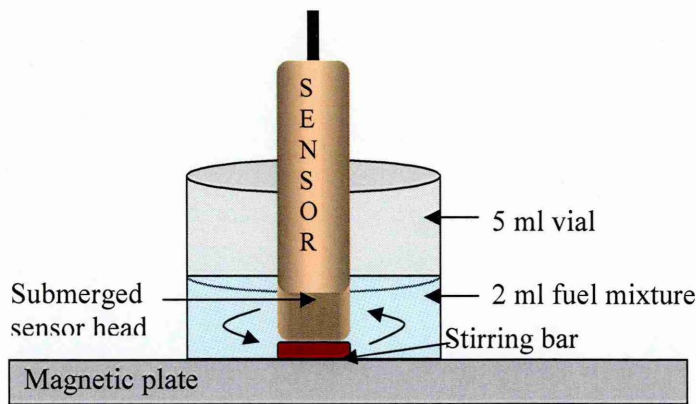


Fig. 3.1 Experimental design to determine Mk 1 sensor responses to sheared fuels.

Two Mk 1 sensors (2.4) were tested simultaneously using the full sensor head assembly (consisting of the protective mesh, O-ring spacer, 11 mm dia. membrane and threaded cap). Sensors were held in place using a clamp and boss during the 30 s sampling time.

3.2.2.2 Detection of water surface fuel contamination

Two open-topped Pyrex glass petri dishes (75 mm dia.) were filled with 50 ml of RO water. Using a clamp & boss, a Mk 1 sensor was impinged upon the surface layer of each, in either of two sensor-surface orientations; a) sensor body vertical – membrane parallel to surface, b) sensor body at 45° to surface – membrane half-submerged.

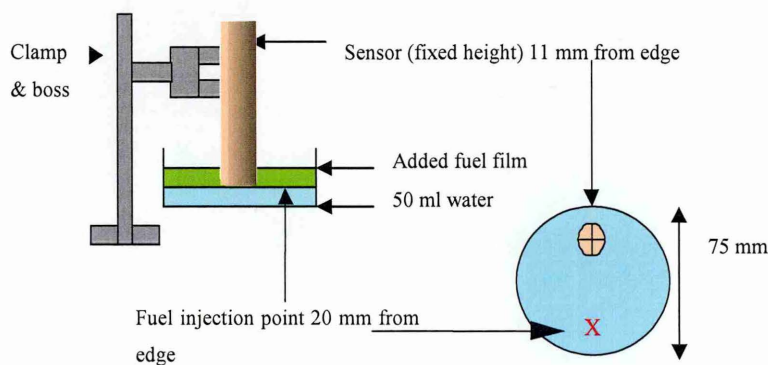


Fig. 3.2 Experimental arrangement for water surface experiments

The vertical positioning of the sensor is shown with the sensor head resting on the surface film. The sensor head placement in relation to the fuel injection point (X) is indicated.

To ensure accurate and reproducible positioning of the sensor head, an 11 mm dia. circle was drawn on the base of each petri dish, 11 mm from the wall. The PETROLog programme was sampled at 1 s intervals, after an initial 5 s period given to establish baseline signals. Fuel was then gently pipetted onto the water surface, 20 mm from the front edge of each petri dish.

Sensor modifications

Two modifications were made to the Mk 1 sensor (Fig. 3.3) during the water surface experiments (Table 3.1); A) Exps. 1-4: the protective mesh was removed; B) Exps. 5 & 6: the entire head assembly was removed and a 20 mm dia. Fluorotrans membrane was placed directly over the glass face of each sensor, in order to completely expose the membrane. This was flush to the glass sensing window (of the sensor body) and held in place with an O-ring. Full experimental parameters are shown in Table 3.1.

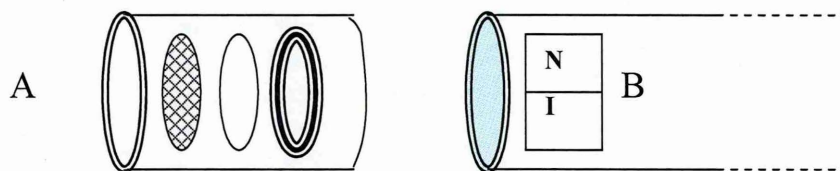


Fig. 3.3 Diagram of the full sensor head assembly.

A) Hollow sensor head containing (left to right) protective mesh, 11 mm dia. Fluorotrans membrane, rubber O-ring; B) Glass sensing window (covering the NIR diodes) of the sensor body.

Table 3.1 Experimental parameters for water surface contamination experiments

The following table shows a summary of experimental parameters for this series of experiments.

¹ Original Mk 1 sensor head assembly consisted of (in order): threaded cap, protective mesh, 11 mm dia. membrane disc, O ring spacer. This then fitted onto the glass sensing window (containing the NIR diodes) of the sensor body (Fig. 3.3).

Exp. No.	Fuel type	Fuel additions added (μl)	Addition interval (s)	Sensor position & membrane position	Head Assembly ¹	Stirred
1	Petrol	100	10	Vertical Surface	No mesh	No
		100	50			No
2	Diesel	100	10	Vertical Surface	No mesh	No
		100	50			No
3	Petrol	100	10	45° angle 50% submersion	No mesh	No
		100	20			No
4	Diesel	100	10	45° angle 50% submersion	No mesh	No
		100	20			No
		100	5			Yes
5	Diesel	100	10	45° angle 75% submersion	Membrane only	No
		100	40			No
		-	5			Yes
6	MTBE	100	10	45° angle 75% submersion	Membrane only	No
		100	20			Yes

3.2.3 Evaluation of sensitivity and dynamic range of alternative membranes

In these experiments, fuel was added directly to dry sensor membranes. The following membranes were evaluated: Biodyne Nylon 6,6, Emflon PTFE, Fluorotrans PVDF, GHP450 (GH polypro), Presense, Versapor R membrane (from Pall Europe Ltd., Portsmouth, UK) and Mycelx (Gainesville, USA). Membrane specifications are given in Appendix 2.

Two Mk 3 sensors (2.5.3) were held in a clamp & boss within a fume hood. Membranes (20 mm dia.) were held flush to the sensor face using an O-ring. The head assembly was not used (Fig. 3.3). After each evaluation, the membranes were discarded and the glass sensing windows were cleaned thoroughly with methanol. The PETROLog programme sampled every 1 s. Baseline values were established for ~10 s for each membrane tested to determine the degree of initial opacity. After this time, 16 µl of fuel was pipetted centrally onto the membrane surface. Responses were monitored for ~50 s.

3.2.3.1 Membrane response to repeat contamination and regeneration by evaporation in air

Regeneration tests were conducted on Fluorotrans, polypropylene and PTFE with unleaded petrol and diesel.

3.2.3.1.1 Petrol contamination

After establishment of a stable baseline (10 s), 8 µl of unleaded petrol was then pipetted onto the membrane surface and the response monitored for 60 s. Three further aliquots (8 µl) were added to the membrane at 60 s intervals to investigate membrane loading capacities. Monitoring was then continued to determine whether the signal returned to near-baseline values, indicating membrane regeneration. Additional aliquots of petrol (8 µl) were added *if* membrane regeneration occurred to determine the repeatability of membrane response (Table 3.2).

Table 3.2 Further experimental parameters for membrane regeneration after petrol contamination by evaporation in air.

Column 2 shows the frequency and time each aliquot (8 µl) was added to the membrane. Where regeneration was not complete, the signal (V) at the time of the additional fuel additions are given in column 4. The time taken for regeneration is shown in column 5.

Membrane type	Time & frequency of fuel aliquots (min)	Baseline signal (V)	Regeneration signal (V)	Recovery time (min)
Fluorotrans	6, 8, 11, 14, 18	0.2	0.2	2, 3, 3, 4
Polypropylene	6, 11, 21, 31, 41	0.2	0.275	5, 10, 10, 11
PTFE*	None	1.15	0.95V	30

* No further aliquots were added to the PTFE membrane.

3.2.3.1.2 Diesel contamination

Experiments were initially conducted as with petrol. After establishment of a stable baseline (10 s), 8 μ l of diesel was then pipetted onto the membrane surface and the response monitored for 60 s. Three further aliquots (8 μ l) were added to the membrane at 60 s intervals to investigate membrane loading capacities. Monitoring was then continued. However, no further aliquots were added to any of the membranes. Monitoring was later extended for a further 35 min period (1 s sampling) and again with 5 s sampling over 25 hr.

3.2.3.2 Membrane response to repeat contamination and regeneration by dissolution in water

Regeneration tests were conducted on Fluorotrans, polypropylene and PTFE with unleaded petrol and diesel. For each experiment, contaminated sensors were immersed into a beaker containing 400 ml RO water, which was moderately stirred (300 rpm) on a Hanna HI-190M magnetic stirrer (Hanna Instruments, Leighton Buzzard, UK) using a 3 x 0.5 cm stirring bar.

3.2.3.2.1 Petrol contamination

After establishment of a stable baseline (10 s), 8 μ l of fuel was then pipetted onto the membrane surface. In contrast to 3.2.3.1, the sensor head/membrane was immediately immersed in a beaker containing 400 ml RO water, stirred at 300 rpm (3.2.3.2). Sensor response was monitored for 60 s. Three further aliquots (8 μ l) were added to the same membrane at 60 s intervals (for this and subsequent contaminations, the sensor was removed from the water, the membrane re-contaminated, then the sensor was immediately replaced). Monitoring was then continued. Additional aliquots of petrol (8 μ l) were added *if* membrane regeneration occurred to determine the repeatability of membrane response (Table 3.3).

Table 3.3 Further experimental parameters for membrane regeneration after petrol contamination by dissolution in water.

Column 2 shows the frequency and time each aliquot (8 μ l) was added to the membrane. Where regeneration was not complete, the signal (V) at the time of the additional fuel additions are given in column 4. The time taken for regeneration is shown in column 5.

Membrane type	Time & frequency of fuel aliquots (min)	Baseline signal (V)	Regeneration signal (V)	Recovery time (min)
Fluorotrans	7, 11	0.2	0.4	7, 11, 22
Polypropylene	None	0.2	2.18	14 *
PTFE	None	1.0	None	None

* A small indication of regeneration occurred with the polypropylene membrane 2.4 V down to 2.18 V after 14 min but no further regeneration occurred during an extended experimental time of 5 hr.

3.2.3.2.2 Diesel contamination

After establishment of a stable baseline (10 s, 8 μ l of diesel was pipetted onto the membrane surface and the sensors immediately immersed in a beaker containing 400 ml RO water, again stirred at 300 rpm (3.2.3.2). Sensor response was monitored for 60 s. The sensor was removed and a further aliquot of diesel (8 μ l) was added to the membrane and the sensor immersed back into the water. No further diesel was added to either membrane. Sensor signals were monitored (1 s sampling) for 35 min. This was then extended (5 s sampling) over 24 hr.

3.2.4 Evaluation of the multi-channelled sensing system for continuous monitoring of fuel in soil substrates (laboratory-based leaking UST simulations)

An in-house tank system was designed for a series of sensing scenarios in order to evaluate the multi-channelled sensor's response to fuel contamination within soil. A glass aquarium (Peak Aquatics, Buxton, Derbyshire, UK) measuring 45.7 (l) x 25.4 (w) x 20.0 (h) cm was used as the holding tank. A standard 250 ml plastic conical flask was used to represent an underground storage tank (UST) and hold the fuel. A 1 mm dia. hole was drilled in the base 1 cm from the side of the flask to enable the fuel to leak out into the tank (Fig. 3.4. The leakage rate of this simulated UST was 20 ml/min in unhindered conditions (no water or sand surrounding the flask).

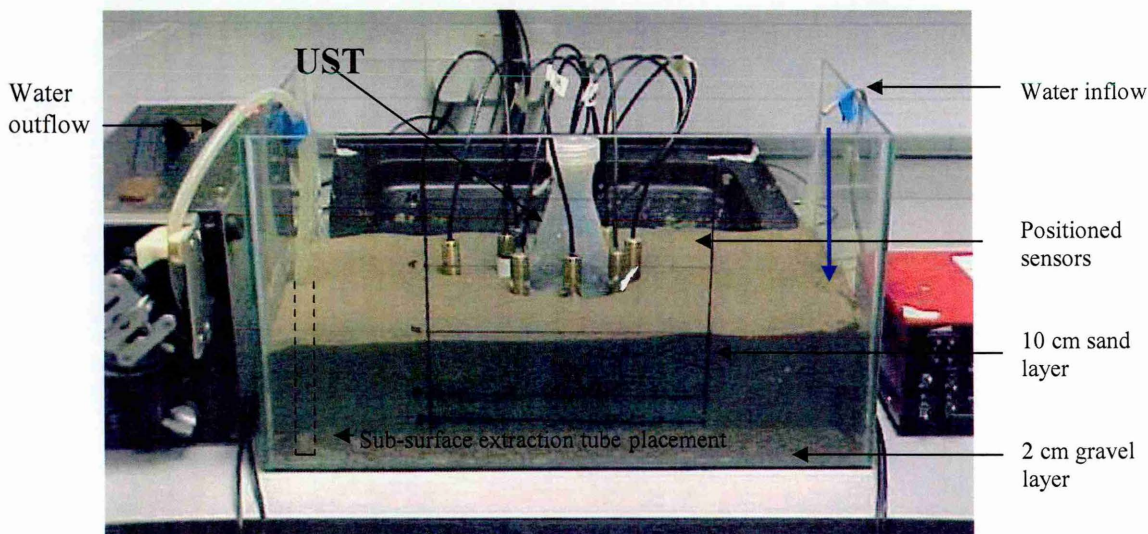


Fig. 3.4 Experimental arrangement for laboratory-based leaking UST simulations

A 250 ml volumetric flask was used to simulate an underground storage tank. The UST flask and sensor positions were varied according to each experiment's objectives (Table 3.5). The tank is shown *partially* filled (depth of 9 cm sand over 2 cm gravel) with the sensor array in place around the buried UST.

The multi-channelled sensor array consisted of seven Mk 3 sensors and two Mk 2 sensors (2.5.2). The full head assemblies were removed from the Mk 2 sensors. Instead, a 20 mm dia. Fluorotrans membrane was placed directly over the glass sensing window by an O-ring. The

MK 3 sensors were used with the head assembly in place. Over a 2 cm base gravel layer, the tank was filled with sand (Table 3.4) to a *total depth* of 12 cm, unless otherwise indicated. The sensor array was placed in various configurations around the UST as the tank was filled. Sensors were activated and the responses signals recorded hourly using the PETROLog programme (2.6.1). The work is summarised in Table 3.5.

3.2.4.1 Groundwater flow simulation

To imitate groundwater movement, water inflow was provided through 3 mm (inner dia.) neoprene tubing (Fisher Scientific UK Ltd, Loughborough, UK) connected to a Watson Marlow (Type 101 U/R) peristaltic pump, which enabled a variable water inflow rate of between 0 -17.6 ml/min (Fig. 3.4). Water was extracted using 10 mm inner dia. flexible plastic tubing, connected to a Watson Marlow (Type 501) peristaltic pump. When the extraction tube was placed in water, 10 rpm gave an extraction rate of 20 ml/min. However, in these experiments, the tube was immersed in the gravel bed (Fig. 3.4) at the base of the tank – the purpose of the pumping being to draw water through the bed, simulating groundwater flow. Consequently, the water removal rate was very much less than this and at the beginning of most experiments, only air was pumped out. Full experimental variables are provided in Table 3.5.

Table 3.4 Sand & gravel used for leaking UST simulations.

Over a 2 cm (depth) gravel drainage layer, the tank was filled to a total depth of 15 cm with sand for each investigation. Four commercial materials were used.

Make	Type	Texture
SupaMix	Path gravel	20 mm dia. coarse cut gravel.
SupaMix	Building sand	Damp. Medium, homogenous grains. Tendency to clod.
Mix-It	Kiln-dried block paving sand	Very dry. Fine homogenous grains.
SupaMix	Sharp sand	Damp. Coarse, mixed grain & small ~1 cm stones.

Table 3.5 Experimental variables for leaking UST simulations

Soil water content was controlled by varying the water inflow and extraction rates.

Method section	Sand type	Sand Condition ¹	Inflow rate [ml/min]	Extraction rate [rpm] ²	Fuel	Fuel leaked [ml]	Time [hr]
3.2.6.1	Building	Wet	0	10	Diesel	200	24
3.2.6.2.1	Kiln dried	Saturated	4	10	Diesel	100	4
3.2.6.2.2	Kiln dried	Dry	8	0	Diesel	100	5
3.2.6.2.3	Kiln dried	Dry	4	10	Diesel	100	5
3.2.6.3	Kiln/Sharp	Damp	0	10	Diesel	100	3
3.2.6.4	Sharp	Damp	8	10	Diesel	100	1.4
3.2.6.5	Sharp	Damp	8	10	Petrol	100	2
3.2.6.6	Sharp	Damp	8	10	Diesel	100	17

¹ Visual assessment ² An extraction pump rate of 10 rpm (20 ml/min) allowed no water accumulation within the gravel layer or on the surface of the sand during these experiments.

Contaminated sand, gravel and sensor membranes were discarded after each experiment. The tank, sensors and flask were thoroughly washed in Teepol (5% v/v) detergent, then thoroughly cleaned with methanol and allowed to dry. Tubing was flushed through with a mixture of water/detergent and rinsed with tap water. All waste materials were disposed of according to current environmental practices.

3.2.4.2 Preliminary determination of optimum sensor depth positioning in wet sand

Mk 3 sensors were used for this determination. The tank was flooded with 4 l water and 1 l excess surface water was removed (using the extraction pump). Four Mk 3 sensors were placed vertically (sensing-face down) at different depths and distances from the UST leak point (Fig. 3.5). Sensor monitoring was started and the UST vessel filled with 200 ml diesel.

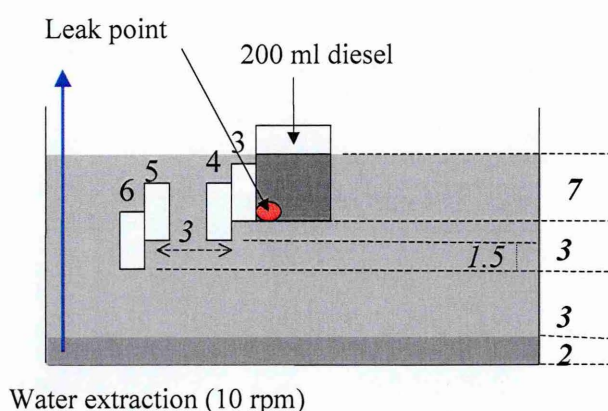


Fig. 3.5 Preliminary determination of sensor depth positioning in wet sand

Sensors (numbered 3-6) were positioned at increasing distances from the leak point and at varying depth, indicated on the right. All units are in cm. Sensors were sampled hourly for a total time of 4 hr. The UST was filled with 200 ml diesel. Water extraction was set at 10 rpm (3.2.4.1).

3.2.4.3 Determination of circular plume movement around a leaking UST

Fig. 3.6 shows the UST and sensor positioning arrangement used for this investigation. Fuel was leaked into both wet sand (containing 3 l water) and dry sand. Water was subsequently introduced to the dry sand, at different flow rates. The aim was to examine the effect of soil water on sensor sensitivity and determine plume movement around the UST. The peristaltic extraction pump was set at 10 rpm throughout this series of experiments (3.2.4.1), which allowed no water accumulation above the surface of the sand.

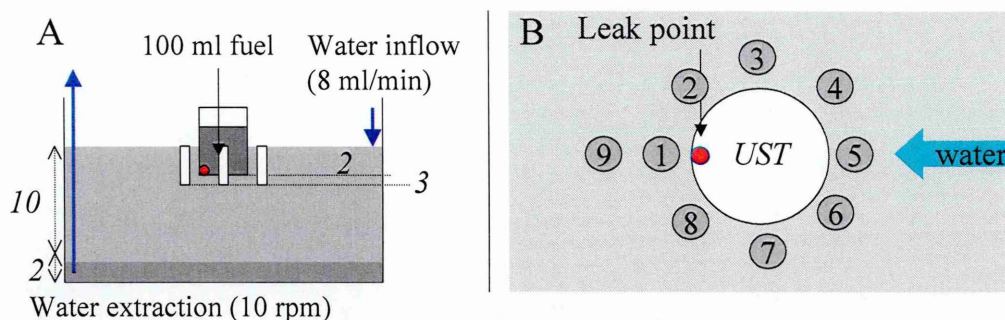


Fig. 3.6 Circular sensor positioning around UST vessel

A) Side view. The UST vessel was sited and buried 2 cm into the centre of the tank and the sensors placed at equal 3 cm depths around it. B) Overhead view. Sensor 9 was placed 3 cm behind Sensor 1. This arrangement was used for experiments described in Sections 3.2.4.3.1-3.2.4.3.3.

3.2.4.3.1 Wet sand

The tank was flooded with 4 l water. An excess of surface water (1 l) was then pumped out using the peristaltic extraction pump. Sensors were placed vertically (sensing-face down) in a circular fashion around the UST vessel (Fig. 3.6). The UST vessel was filled with 100 ml diesel. Initial water inflow of 4 ml/min was discontinued after 30 min (as the tank was over-saturated). The extraction rate (10 rpm) was maintained throughout the experiment (3.2.4.1). The rate of fuel leakage was monitored for 4 hr.

3.2.4.3.2 Dry sand (with extended monitoring)

Two experiments were conducted in dry sand with two water inflow rates, 4 ml/min and 8 ml/min respectively. Water inflow (8 ml/min) was started at the same time as the fuel leakage so that initially the fuel leaked into totally dry sand. Sensors were positioned as shown in Fig. 3.6. The UST vessel contained 100 ml diesel. Both experiments were initially monitored for 5 hr. The water inflow was then stopped and monitoring continued for 48 hr. The results were combined (4.2.4.3).

3.2.4.4 Determination of vertical plume movement with a horizontal sensor ‘fan’ formation

At different depths, sets of three sensors were placed horizontally in a ‘fan’ formation around the UST leak point, each 2 cm from the edge of the UST (Fig. 3.7). Mixed sand was used (Kiln and Sharp sand) which was uniformly dampened with 3 l water. The extraction pump was run at 10 rpm (3.2.4.1). For this experiment, there was no water inflow as the aim was to determine the vertical flow of fuel (100 ml diesel) over a 3 hr monitoring period.

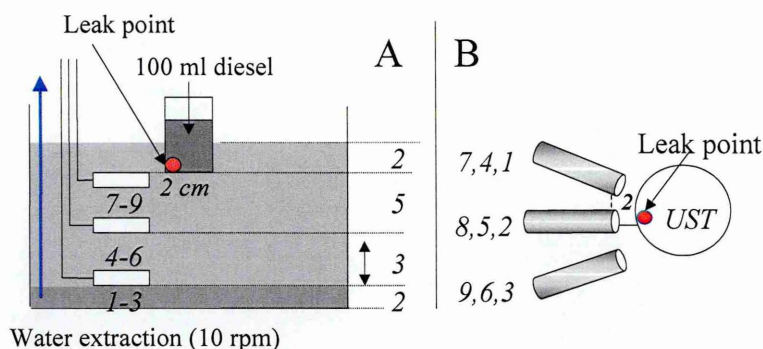


Fig. 3.7 Horizontal placement of Mk 3 sensors at different depths

A) Side view showing each set of 3 'fanned' sensors at increasing depths (2, 7 & 10 cm) beneath the surface. The UST base was placed 2 cm beneath the surface; B) Overhead view showing 'fan' positioning of sensors around the UST leak point. Each sensor was 2 cm apart (head to head) and 2 cm distance from the edge of the UST base. This positioning was repeated at each depth (italics show the top to bottom sensor order).

3.2.4.5 Determination of fuel plume movement with a vertical grid of sensors

To further investigate horizontal fuel plume movement, 9 sensors were arranged in a grid formation and placed deep underneath the UST 1 cm above the gravel layer (Fig. 3.8). The UST vessel contained 100 ml diesel. The extraction pump was run at 10 rpm (3.2.4.1), water inflow was 8 ml/min. Monitoring was for 2 hr. This experiment was repeated using 100 ml standard grade unleaded petrol as the contaminating fuel.

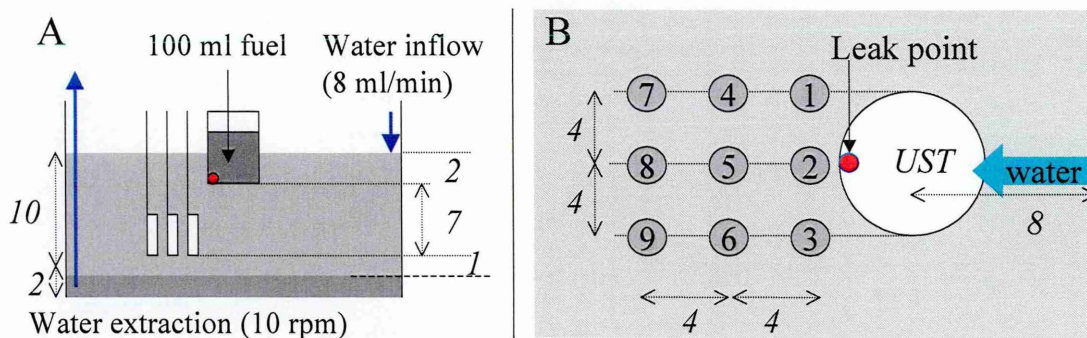


Fig. 3.8 Vertical grid formation

A) Side view showing sensors placed vertically 1 cm above the gravel layer in damp sand. The UST base (and leak point) was 7 cm above the sensor heads. B) Overhead view showing the grid formation of sensors in relation to the leak point. The extraction pump was run at 10 rpm (3.2.4.1), water inflow was 8 ml/min. Monitoring was for 2 hr.

3.2.4.6 Investigation of membrane regeneration through fuel dissolution by soil water movement

A circular sensor arrangement was again used, the UST sited closer to the water inflow point than in previous experiments (Fig. 3.9). Water inflow was 8 ml/min with the extraction pump run at 10 rpm (3.2.4.1). Monitoring was for 17 hr.

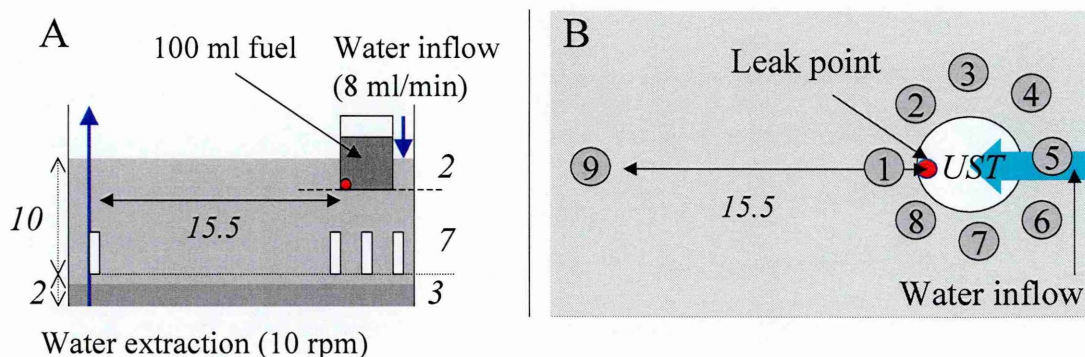


Fig. 3.9 Circular sensor arrangement with sensors positioned at depth

A) Side view. Eight sensors were placed 1 cm from the flask base and 1 cm above the gravel layer (7 cm beneath the UST base). B) Overhead view. Sensor 9 was positioned as far as possible from the UST, 15.5 cm from the leak point. An 8 ml/min water inflow and an extraction pump rate of 10 rpm was maintained throughout.

3.2.5 Quantitative evaluation of sensor responses to fuels in different soils and conditions

Two Mk 3 sensors, fitted with Fluorotrans membranes (11 mm dia.) were used to determine sensor response to 4 different fuels in three soil types with varied water content. Sieved (2 mm mesh) air-dried clay and sand soils (5 g) were wetted with varied amounts of RO water. Water saturation volumes (for each soil type) were calculated by saturating each soil with excess RO water for 30 min. Non-adsorbed (excess) water was carefully removed from the surface with a pipette. The adsorbed water volume was calculated by subtracting the dry weight of the membrane from the wet weight (Table 3.6). Soil/water mixtures (50 & 100% saturated) for sensor testing were subsequently prepared in 30 ml polycarbonate Universal tubes. Mixtures were thoroughly homogenised for 10 min using a hand-held homogeniser (100 ml) with a Teflon pestle.

Diesel, unleaded petrol, central heating oil and pure MTBE were tested. Fuel contamination volumes (in 5 g soil) were 200, 400 & 600 μ l, which approximately equates to 3.4, 6.8 & 10 % (w/w/dry soil) fuel respectively (average SG = 0.85). Each soil was initially contaminated with 200 μ l fuel and thoroughly mixed for 60 s. Tests were performed in duplicate. After a 5 s baseline period, the sensor was pushed into the sample (contaminated with 200 μ l fuel) to a depth of $\sim$ 1 cm and monitored for 150 s (5 s sampling). Monitoring was paused while the sensor

was removed and a further 200 µl fuel mixed into the sample for 60 s. The sensor was then replaced and monitoring continued. This procedure was repeated after 700 s of monitoring and was then monitored for a total time of 900 s.

Table 3.6 Water adsorption volumes of different soils for 100% saturation

Each soil matrix absorbed a different volume of water for 100% saturation (Column 2). The clay soil ² had the largest water adsorption capacity.

Soil type [5 g] ¹	100% saturation volume [ml]	SD	% CV (n=6)
Building sand	1.15	0.04	5.12
Clay sub-soil ²	3.30	0.06	1.85
50% mixture	2.06	0.166	8.1

¹ Six samples of each soil type were used to determine the water saturation volumes. Mean values are shown, all values were within 10% of the mean, n = 6.

² Clay was 2nd horizon local sub-soil with ~ 80% clay content.

3.2.6 Membrane resistance to biofouling

3.2.6.1 Induced membrane biofouling

3.2.6.1.1 Agar plate incubations

Malt extract agar (MEA) plates pH 5.6 were inoculated with 5 organisms (1 pure culture/plate: *Aspergillus ochraceus* (fungi), *Fusarium poae* (fungi), *Penicillium verrucosum* (bacteria), *Pseudomonas fragi* (bacteria) and *Saccharomyces cerevisiae* (yeast) and incubated at 25 °C for 5 days (cultures were obtained from the Mycology Dept., Cranfield University, Silsoe, UK). A single 5 mm dia. plug was taken from each plate using a cork borer and placed into the centre of a fresh agar plate. These were incubated at 25 °C for 14 days. The cultures were then used to inoculate nutrient broth suspensions (3.2.6.1.2).

3.2.6.1.2 Nutrient Broth Suspensions

Oxoid general-purpose yeast extract powder (13 g) and 1 l RO water was mixed until a homogenous nutrient broth (pH 7.2) was produced. The nutrient broth was then sterilised in an autoclave for 25 min at 120 °C. Into 25 ml sterile glass Universal bottles, 10 ml of nutrient broth was added, cooled to 25 °C and inoculated with a pure culture of each organism (from 3.2.6.1.1). Three replicates per organism were made. A single 11 mm dia. Fluorotrans membrane was then placed into each Universal. Due to the hydrophobic nature of the membrane, the membrane was found to float on the broth surface. To ensure good membrane culture contact, the Universal bottles were placed on an automated shaker (120 rpm) and kept at a constant 25 °C for 14 days.

3.2.6.2 Membrane biofouling under natural ground conditions (factorial experiments)

Two soil types were ground and passed through a sieve (2 mm mesh) and kiln-dried for 48 hr at 120 °C. Into sterilised glass Universal bottles, 10 g of each soil mixture was added (Table 3.7). A single 11 mm dia. Fluorotrans membrane was then centrally placed onto the soil surface and covered with a further 10 g soil mixture. Local, unfiltered groundwater (from an on-site groundwater monitoring well, Cranfield University, Silsoe, UK) was weighed and added to each Universal at either 20% or 80% water (w/w). The groundwater was not sterilised in order to preserve any natural microflora present. Each Universal was loosely sealed and covered with foil. These were stored in darkness at either 5 or 25 °C for 6 months. After 6 months, the membranes were removed from the Universals. Excess soil was removed by gently washing each membrane in sterile RO water for 60 s. Membranes were air-dried for 15 min and the surfaces examined under a Raman microscope at ×10 magnification. Membranes were then tested by direct addition of 4 µl diesel and the response compared to a fresh membrane.

Table 3.7 Classification for factorial analysis of natural membrane biofouling

Soil A was second horizon local soil with 80% clay content (Cranfield University, Silsoe, UK). Soil B was standard grade building sand with no measurable clay content. Three parameters were chosen for factorial analysis: % soil A/B, % water content and incubation temperatures of 5 °C and 25 °C (Fig. 3.10). Each combination was performed in duplicate, n=2.

Label	Variable parameters	Label	Variable parameters
A1	80% soil B +20% soil A	A2	20% soil B + 80% soil A
B1	20% water content ¹	B2	80% water content ²
C1	5 °C incubation	C2	25 °C incubation

¹ 80g soil + 20g H₂O ² 20g soil + 80g H₂O

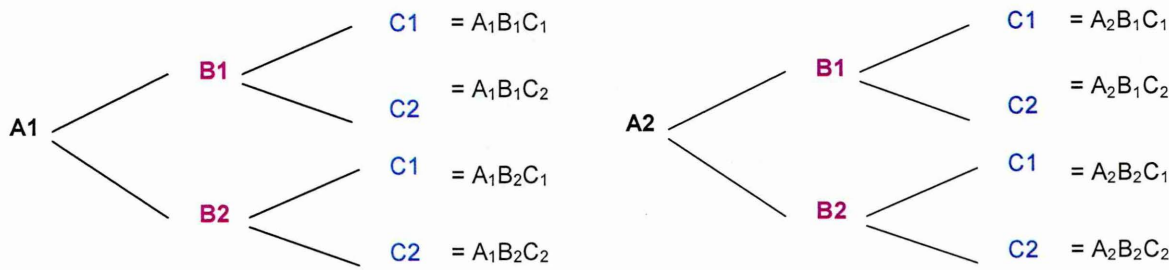


Fig. 3.10 Factorial diagram showing different combinations of experimental parameters.

A1 and A2 refer to soil type (% sand and clay, either 20 or 80%). B1 and B2 refer to the % water content (20 or 80%). C1 and C2 refer to the incubation temperature (5 or 25 °C).

3.2.7 Extraction of BTEX compounds from Fluorotrans membrane

Standard 11 mm dia. Fluorotrans membranes were each contaminated with 8 μ l fuel and immediately placed into a 1.5ml Eppendorf tube containing 1 ml extraction solution - this was either RO water or a 1% (v/v) MeOH solution. The sealed Eppendorfs were placed in a 50 ml glass beaker containing 30 ml spectroscopic-grade H₂O (Sigma-Aldrich, Poole, Dorset, UK) and sonicated for 5 min. The membranes were then discarded and each extraction analysed using the Total BTEX RaPID Assay Test Kit (SDI Inc., USA). Extractions were tested neat and 50% diluted in RO water. The manufacturer's recommended protocol was followed, summarised as follows:

BTEX RaPID Assay Procedure (SDI Inc., USA).

Test tubes were labelled for standards, controls or samples and 200 μ l of each added to the appropriate tube and placed in the SDI (tube) rack. The BTEX enzyme conjugate (250 μ l) was then added to each tube, followed by 500 μ l of BTEX antibody-coupled magnetic particles (beads). Tubes were then gently vortexed for 1-2 s and then incubated at RT (22 °C) for 15 min, and then separated by attaching the SDI magnetic separator for 2 min. Tubes were then decanted, 1 ml SDI washing solution was added to each tube and left for 2 min. This was then removed (by inverting the rack) and the wash step repeated. The (tube) rack was then removed from the magnetic separator and 500 μ l of SDI colour-producing solution was added to each tube. Tubes were vortexed for 1-2s and incubated for 20 min. at RT, where the reaction was then stopped by the addition of 500 μ l SDI stop solution.

At the end of the BTEX IA manufacturer's procedure, 300 μ l of each stopped solution (standards, controls and samples) were put into microtitre wells and the absorbance read at 450 nm (A450). The result was then calculated as % B/Bo and the BTEX concentration [ppm] determined using the SDI supplied graph paper (1.5.3).

3.2.7.1 Membrane extraction of BTEX compounds post-lysimeter field trial (#2)

On termination of the lysimeter field trial #2 (3.3.3), the sensors were immediately excavated. The diesel-contaminated membrane from each sensor was removed and placed into a 1.5 ml Eppendorf containing 1 ml of 1% (v/v) MeOH. Membranes were extracted and analysed using the BTEX RaPID Assay protocol, as described in 3.2.7. A fresh membrane was used as a negative control (NC).

3.2.8 Effects of free-phase unleaded petrol and diesel on plastics

3.2.8.1 Polycarbonate and Polystyrene

Fuel (1 ml) was added to 2 ml RO water contained in either 30 ml polycarbonate sample tubes or 5 ml polystyrene cuvettes (Fisher Scientific UK Ltd., Loughborough, UK), 1 ml fuel was added to 2 ml RO water. The fuel/water mixture was manually shaken for 10 min, sealed with Parafilm and left for 24 hr.

3.2.8.2 Polyethylene terephthalate copolymer (PETg) and Perspex

Strips of both PETg and Perspex were placed into a 10 ml glass beaker containing a mixture of 1 ml fuel and 2 ml RO water. This resulted in 50% coverage of the plastic strips. Each was manually shaken for 10 min, sealed with Parafilm and left for 24 hr.

3.3 FIELD MONITORING TRIALS WITH SUB-SURFACE SENSOR ARRAYS

3.3.1 Field-based data acquisition & transmission

A multiple parameter data-logging unit (DLU), integrated with GSM-based mobile communications (Medsci Consultants UK Ltd, Harpenden, UK) was used for field-based data acquisition and transmission. This was adapted and developed specifically for use with the sub-surface sensors throughout this work (Chapter 2.7).

3.3.2 Preliminary field monitoring investigations using two sub-surface sensor designs for detecting petrol contamination

A lidded, plastic storage box - 32 (h) x 47 (w) x 24 (d) cm, was used as the holding tank. Two Mk 1 sensors (2.4) were evaluated against a prototype cable oil sensor (Fig. 2.3). The sensors were positioned on the base of the tank (Fig. 3.11).

Sensor cables were routed out and connected to the freestanding Medsci DLU via an external junction box (2.7). The tank was filled with medium grade, dry building sand to a depth of 12 cm. The tank was initially contaminated with 50 ml unleaded petrol (directly above sensor 1). Further contamination followed at the same addition point. Full experimental parameters are shown in Table 3.8. The Medsci DLU was programmed and activated to continuously monitor the sensors. During this experiment, the DLU was additionally reprogrammed *in-situ* using the 'OilProbe.exe' programme (2.6.2.1) loaded onto a laptop computer.

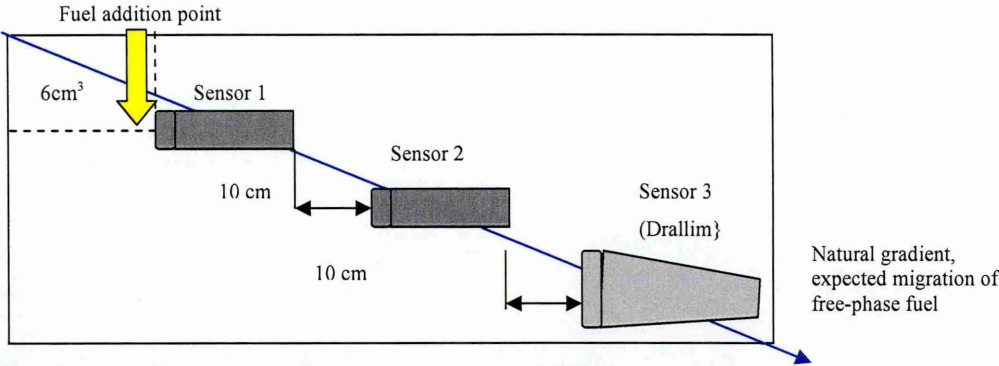


Fig. 3.11 Positioning of three early sensors within a contained tank

The first sensor was placed at the rear left-hand side of the tank, directly beneath the fuel addition zone and 6 cm from each side of the tank (overhead view and not to scale).

Table 3.8 Experimental parameters for preliminary field trial

The table shows the sampling rate, fuel contamination information and monitoring periods for the preliminary field trial using Mk 1 fuel sensors.

Stage	Sampling rate (min)	Email data (hr)	Monitoring period (Day)	Fuel addition	Volume of fuel (ml)
1	15	12	3	30 min	50
2	30	12	7	Day 8	50
3	30	12	9	Day 9	50
4	30	12	12	Day 12	35*

** Small wells were dug to a depth of approximately 2 cm above each sensor head for the final addition of 35 ml petrol per well. Total petrol added to the tank was 205 ml.*

3.3.2.1 Evaluation of Mk 1 Sensors in conjunction with the field-based Medsci DLU under controlled conditions

The sensors & Medsci DLU were moved into a laboratory to investigate the relationship between the cyclic signal variations. The Mk 1 sensors were cleaned in methanol and refitted with a new membrane. The sensor signals were monitored under ambient laboratory temperature (22 °C), sampling every hour, using the Medsci DLU.

3.3.3 Lysimeter-based field monitoring trials with Mk 3 sensor arrays

Long-term, outdoor monitoring trials were designed for evaluating an array of the redesigned Mk 3 sub-surface sensors. A 1 (h) × 0.8 (dia) m lysimeter was designed and built specifically for this purpose (Fig. 3.12). The Fiberdur ® lysimeter body, (Fibaflo Ltd., Poole, UK) was fabricated from epoxy resin and filament-wound, hot cured glass fibre rovings. In one side of the lysimeter, a vertical line of 5 cm dia. holes were drilled at 10 cm intervals to provide sensor cable exits. These holes were covered (to prevent any leakage of soil and/or fuel contaminated water) with rubber and waterproof epoxy sealant. The lysimeter base was sealed with Quent underwater metal (Boston Chemical Co., Wetherby, UK). A 2 cm layer of pea gravel was laid

for drainage. The lysimeter was then filled with sand or soil, with sensors placed intermittently. (Fig. 3.12). The open top design allowed natural flow through of rainwater.

A 19 (h) × 16 (dia) cm cylindrical metal container, with a 6 mm dia. side hole (leak point), 2 cm above the base, represented the UST. This gave a leakage rate of 120 ml/min in unhindered conditions (no surrounding water or soil). In a typical trial, seven Mk 3 sensors were used: three sensors were horizontally placed within a 2 cm layer of gravel supporting the base of the leaking UST vessel. A further 4 sensors were placed horizontally at increasing 10 cm depths (Fig. 3.12).

The Mk 3 sensor cables were routed out of the lysimeter and linked up to the Medsci DLU (Fig. 3.13). This was programmed to activate the sensors and collect the sampling data hourly, with collected 24-hour data sent via email daily at a specified time throughout the monitoring period. Four full lysimeter tests were performed. Full experimental parameters are detailed in Tables 3.9 & 3.10.

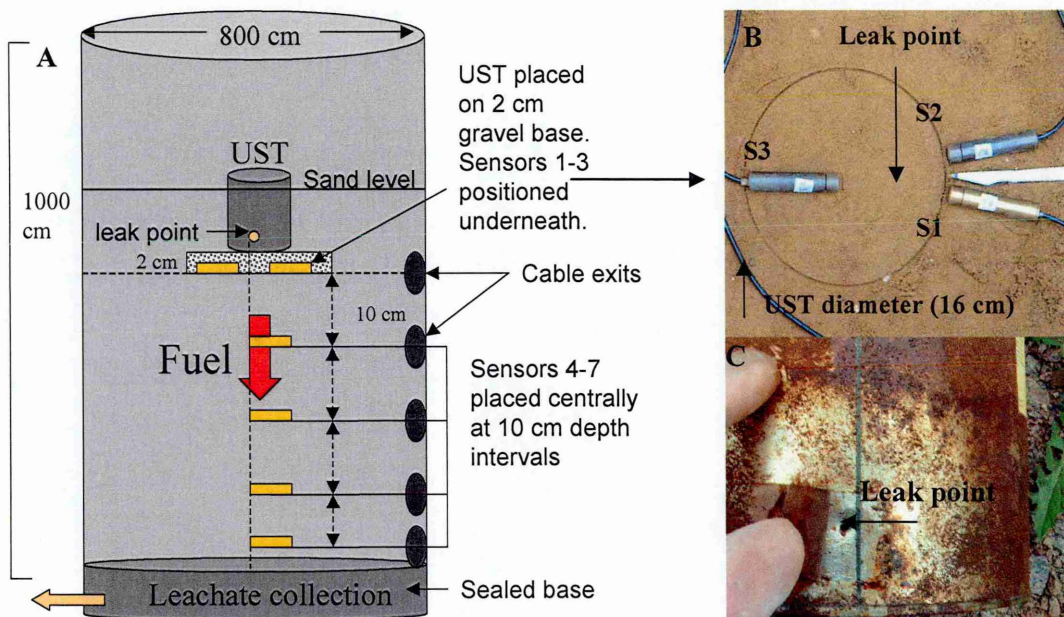


Fig. 3.12 Generic experimental design for the lysimeter field trials

A) Three Mk 3 sensors were positioned directly under the UST vessel. A vertical line of four sensors was arranged beneath these (and centrally beneath the overhead UST) at 10 cm depth intervals. Sensors were emplaced as the lysimeter was filled. Sensor cables were routed out through sealed 5 cm cable exits and connected to the on-site Medsci DLU (Fig. 3.13). B) Positioning of Mk 3 sensors 1-3 directly under the UST base. C) Leak point (UST) showing gauze covering.

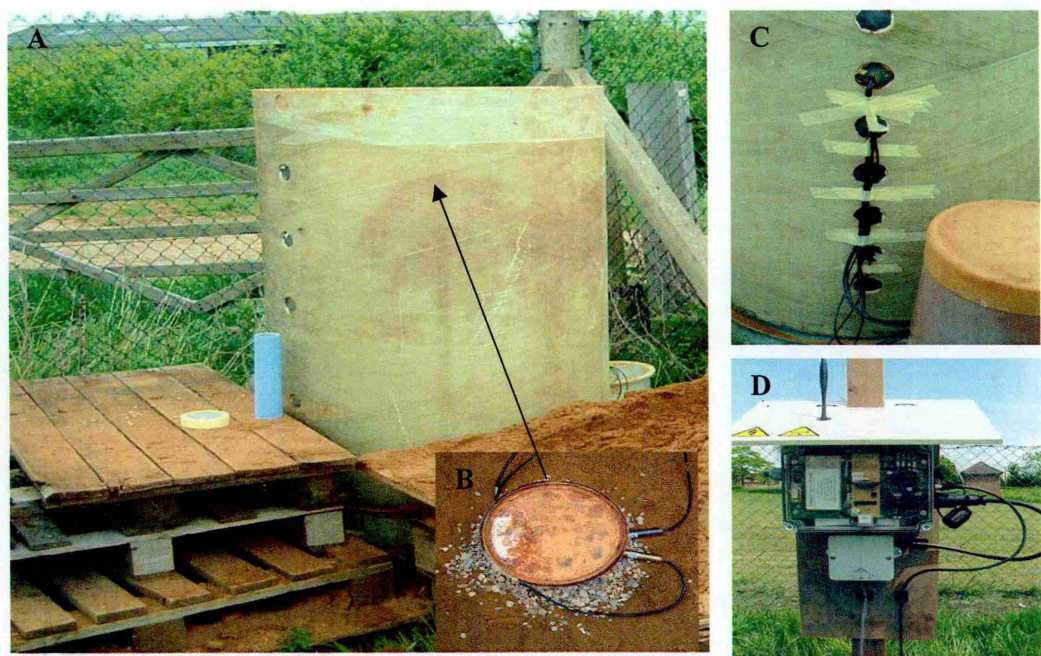


Fig. 3.13 Lysimeter *in-situ*
A) Lysimeter body. B) Insert shows the buried UST and top sensors in place within gravel layer (only sensors 1 and 2 are visible, sensor 3 was central underneath the UST base). The arrow indicates the approximate positioning of the UST within the lysimeter. C) Sensor cable routing from lysimeter to the DLU. D) The Medsci DLU *in situ* (no sensors attached).

Table 3.9 Experimental parameters for the lysimeter field trials

A usual trial format would involve a 1 week stabilisation period, after which 0.5 l of contaminant would be leaked from the UST (1 l fuel would be added to the UST and 0.5 l would leak from the leak point in the side of the UST). Additional fuel was leaked at various stages, noted for each trial. Additional experimental variations are shown in Table 3.10.

Trial No.	Soil type	Fuel type	Additional fuel/time passed (l/date)	Total fuel leaked (l)	Dates (month/yr)	Monitoring period (wk)
1	Sand	Diesel	0.5/14.10.02 0.5/15.10.02	1.5	Oct - Nov 02	4
2	Sand	Diesel	0.5/30.5.03 0.5/12.6.03 0.5/19.6.03 0.5/03.7.03	2	May - Aug 03	12
3	Topsoil	Petrol	0.5/19.4.04	0.5	April - May 04	5
4	Topsoil	Diesel	0.5/11.6.04 0.5/ 25.6.04	1	June - Aug 04	5

Table 3.10 Additional experimental variations

Additional experimental variations during the lysimeter-based field monitoring trials are shown.

Trial	Variable
1	The two sensors either side of the leak point (s1 and s3) were spaced 10 cm apart instead of 2 cm (head to head). These sensors did not use the full head assembly (Fig. 3.3). Instead a 20 mm dia. Fluorotrans membrane was attached over the sensor body.
2	Membrane extraction experiment conducted (3.2.7).
3	A faulty Mk 3 sensor (7) was replaced with Mk 2 sensor (also without the full head assembly).

CHAPTER 4

RESULTS (Sub-surface monitoring)

4.1 INTRODUCTION

The development of the sub-surface monitoring system has been an integral part of this project. This chapter describes the results of the experimental work conducted with the sub-surface sensors. The chapter is divided into two main parts. The first part relates to the extensive laboratory evaluations of the sub-surface sensors (Section 4.2). These results directly contributed to continuous improvements to the *design* of the sub-surface sensors and data acquisition systems, which are described in Chapter 2. The second part of this chapter (Section 4.3) describes the results from field monitoring trials with the improved Mk 3 sub-surface monitoring system as a complete unit. The chapter ends with a general discussion (Section 4.4). For clarity, these results have been summarised chronologically in Table 4.1 and cross-referenced to the appropriate section. Detailed results are reported thereafter.

Table 4.1 Summary of sub-surface sensor evaluations

The table shows the order of presented results, together with the appropriate results and methods sections.

Results section	Evaluation	Methods section
4.2.1	Detection of sheared fuels in water	3.2.2.1
4.2.2	Detection of water surface fuel contamination	3.2.2.2
4.2.3	Evaluation of alternative membranes	3.2.3
4.2.4	Laboratory monitoring (UST simulations)	3.2.4
4.2.5	Sensor responses in different soils	3.2.5
4.2.6	Membrane resistance to biofouling	3.2.6
4.2.7	Extraction of BTEX from membranes	3.2.7
4.2.8	Alternative sensing window materials	3.2.8
4.3	Field monitoring trials	3.3
4.3.1	Preliminary field evaluation of Mk 1 sensors	3.3.2
4.3.2	Multi-channel lysimeter-based field trials (4)	3.3.3

4.2 LABORATORY EVALUATIONS FOR SUB-SURFACE SENSOR DEVELOPMENT

4.2.1 Evaluation of the MK 1 sensors to neat fuels & sheared fuels in water

The Mk 1 sensors were evaluated as potential devices for the detection of hydrocarbon fuels (HCF) in water (3.2.2). Sensor response was determined by the sensitivity of the membrane to contaminants present either in solution or suspended in the water matrix.

Fig. 4.1 shows that the Mk 1 sensors reached an average maximum signal of ~ 2.25 V in undiluted fuels and responded equally well to oil, petrol, diesel and MTBE. Baseline (uncontaminated) values varied by ~ 0.2 V between individual sensors and were between 1.05-1.25 V. Complete translucency of the Fluorotrans membrane could be achieved with 8 μ l fuel. The membrane also responded to organic solvents - methanol, ethanol, acetone & 1-propanol (not illustrated). The Mk 1 sensors responded equally to sheared diesel & petrol in water and also MTBE solutions down to a 20% (v/v) fuel/water concentration, again reaching a maximum of ≥ 2.25 V. A slower (possibly cumulative) response was sometimes evident with 20% petrol (Fig. 4.2) and diesel mixtures. Weaker mixtures of these fuels were not detected. However, MTBE solutions were detected at 10% (v/v).

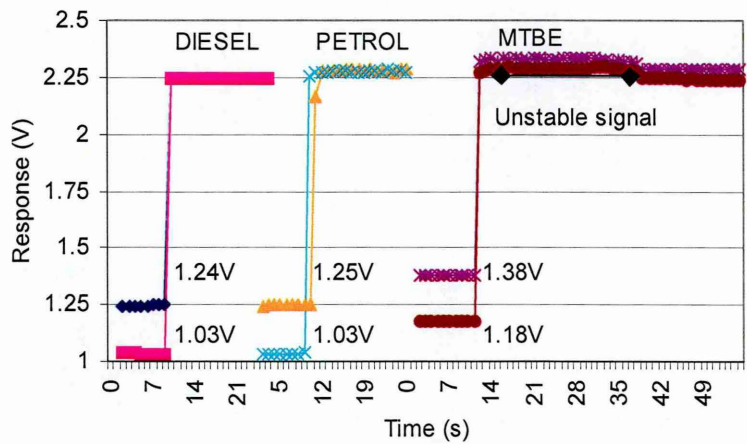


Fig. 4.1 Mk 1 sensor response to undiluted fuels

Two Mk 1 sensors were tested with neat fuels (3.2.2.1). Once a 5 s baseline was established, sensor heads were submerged in each fuel mixture. The numbers 1 & 2 refer to sensors 1 and 2. The MTBE evaluation was extended to 50 s to establish a stable maximum signal.

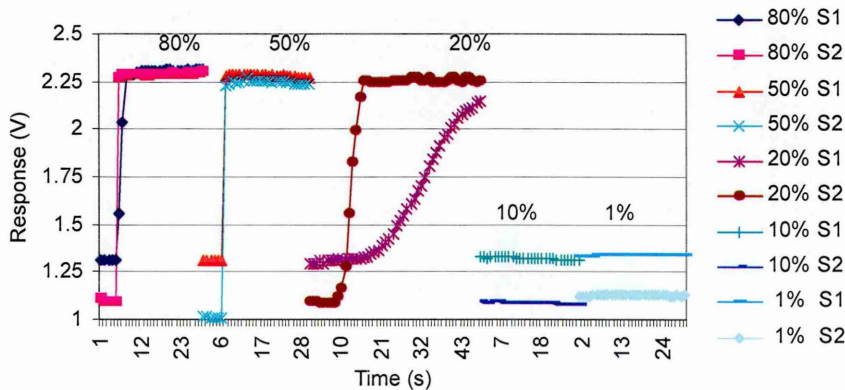


Fig. 4.2 Mk 1 sensor response to unleaded petrol in water

Two Mk 1 sensors were tested with different sheared diesel/water mixtures, as indicated by the percentage concentration (3.2.2.1). S1 and S2 within the legend key refer to sensors 1 and 2 respectively.

4.2.2 Evaluation of MK 1 sensors for detection of water surface contamination by free-phase fuels

The MK 1 sensors were evaluated as potential passive monitors to detect fuels present on a water surface. Organic fuels are less dense than water and so, early free-phase contamination could be expected to accumulate on the water surface. Methods are described in 3.2.2.2. A summary of the water surface contamination experiments is shown in Table 4.2.

Table 4.2 Experimental parameters and summary of results for the detection of fuel contamination on water surfaces. The sensor head assembly¹ was modified throughout these experiments, as detailed in Column 6. ¹Original sensor head assembly consisted of: a threaded cap, protective mesh, O ring spacer and a 11 mm dia. membrane disc. This then fitted in reverse order onto the glass housing (containing the NIR emitter/receiver) of the sensor body (Fig. 2.7, Section 2.4.2).

Exp. No.	Fuel	Fuel increments added [μL]	Time (s)	Sensor position & % submersion	Head Assembly ¹	Stirred	Sensor response & max. signal [V]
1	Petrol	100	10	Vertical 1%	No mesh	No	No
		100	50			No	No
2	Diesel	100	10	Vertical 1%	No mesh	No	No
		100	50			No	No
3	Petrol	100	10	45° angle 50%	No mesh	No	No
		100	20			No	2.05
4	Diesel	100	10	45° angle 50%	No mesh	No	No
		100	20			No	1.10
		100	5			Yes	No
5	Diesel	100	10	45° angle 75%	Membrane only	No	No
		100	40			No	No
		-	5			Yes	2.36
6	MTBE	100	10	45° angle 75%	Membrane only	No	No
		100	20			Yes	No

4.2.2.1 Vertical positioning of the Mk 1 sensors

The Mk 1 sensors did not detect water surface contamination (a maximum of 200 μl fuel/44.2 cm²) when vertically held on the water surface. The fuel surface film was clearly visible, reaching the sensor heads after 2-3 min., yet did not contact the membrane, passing around the sensors in a circular motion. The sensor head depressed the water surface so that the membrane was lower (deeper) than that of the surrounding water/fuel mixture. It was thought this may have caused an air barrier across the membrane surface, or alternatively, water acted as a fuel-repelling film around the sensor head preventing contact with the membrane.

4.2.2.2 Sensors positioned at 45 ° and semi-submerged (50%) beneath the water surface

Altering the sensor angle to 45 ° and semi-submerged (50%) beneath the water surface did not improve the sensors' ability to detect water surface fuel contamination. It was thought that

angling the sensors into the water may have increased the likelihood of some fuel contact through decreased surface displacement. Removing the entire head assembly and placing a larger 20 mm dia. membrane directly over the glass sensing window (3.2.2.2) had no effect. A single accumulative response to both petrol and diesel (200 μ l) occurred after splashing during stirring and repeated immersion beneath the surface (Fig. 4.3-4.4).

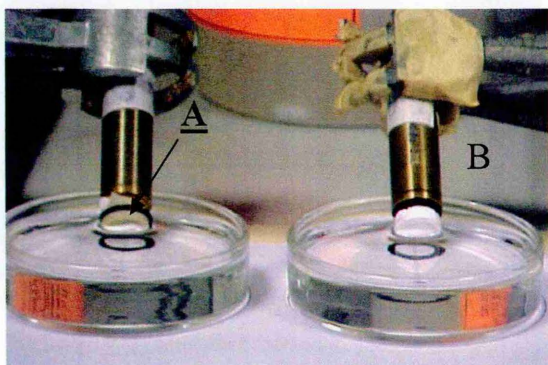


Fig. 4.3 Mk 1 sensors held at 45 ° semi-submerged (50%) in water

A) Membrane translucency with 200 μ l diesel as a result of splashing - the opaque membrane under the surface can be clearly seen; B) The second sensor did not respond after stirring.

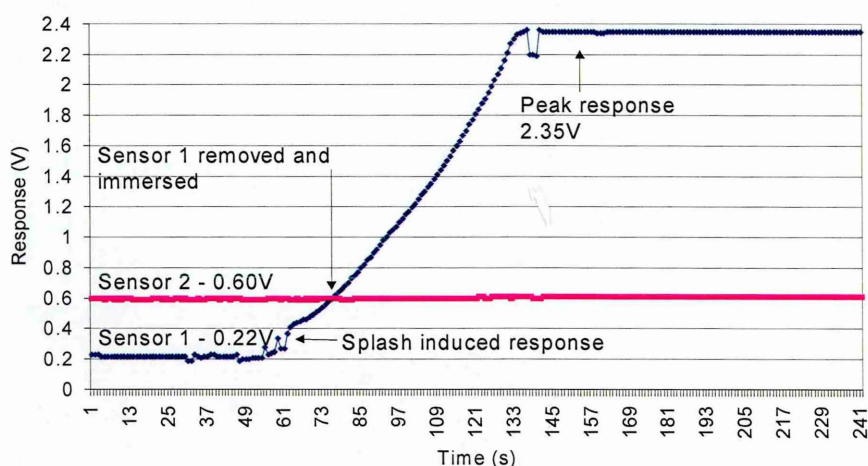


Fig. 4.4 Induced response to 200 μ l diesel surface contamination

The surface was vigorously agitated between 50-70 s. After 60 s sensor 1 was repeatedly immersed vertically beneath the surface. Sensor 2 was not manipulated.

4.2.3 Evaluation of alternative membranes for use with the sub-surface sensors

4.2.3.1 Evaluation of sensitivity and dynamic range of alternative membranes

Alternative membranes with different absorption and filtration properties were evaluated for potential use with the sub-surface sensors. These were selected due to their different properties and their commercial availability (Appendix 2), the aim to select membranes exhibiting the most suitable properties with respect to detection of HC fuels. Each membrane was tested in turn with three common fuels: central heating oil, diesel (DERV) and unleaded petrol (3.2.3), which was pipetted (neat) directly on to the membrane surface.

Fig. 4.5 shows that maximum signals (≥ 2.4 V) were obtained with Fluorotrans and PTFE in response to central heating oil, although the PTFE exhibited an undesirably high baseline signal indicating a high degree of light transmission. Good responses (>2 V) were shown by Polypropylene and GHP450 membrane, although these were more variable. The Versapore membrane did not absorb the oil. Fluorotrans also produced the highest response to diesel contamination (Fig. 4.6). GHP450, polypropylene and PTFE also had good responses ~ 2.3 V although more signal fluctuation was observed. Responses were much more variable with petrol contamination (Fig. 4.7). Fluorotrans had the most consistent maximum response. All membranes (except PTFE) showed a potential for regeneration after exposure to petrol, indicated by the gradual fall in signal.

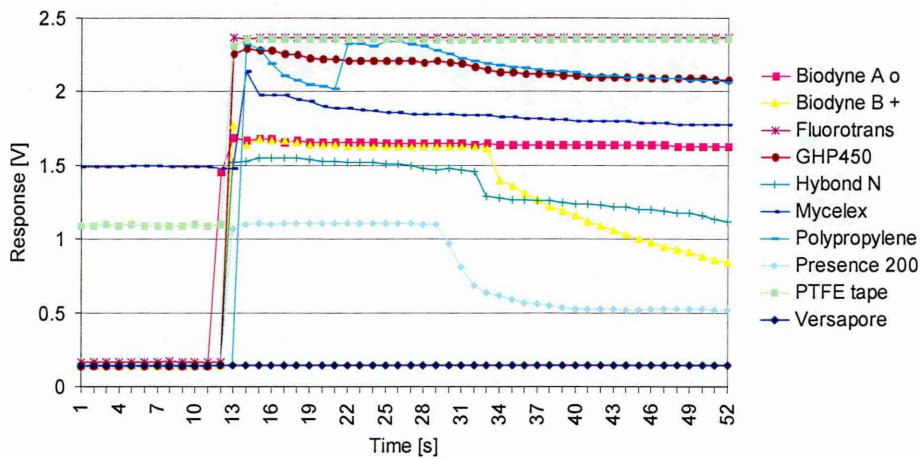


Fig. 4.5 Membrane responses to central heating oil

Membranes (20 mm dia.) were fixed over the glass sensing window of a Mk 3 sensor (3.2.3). After a ~ 10 s baseline period, 16 μ l central heating oil was pipetted onto the membrane and monitored for ~ 50 s.

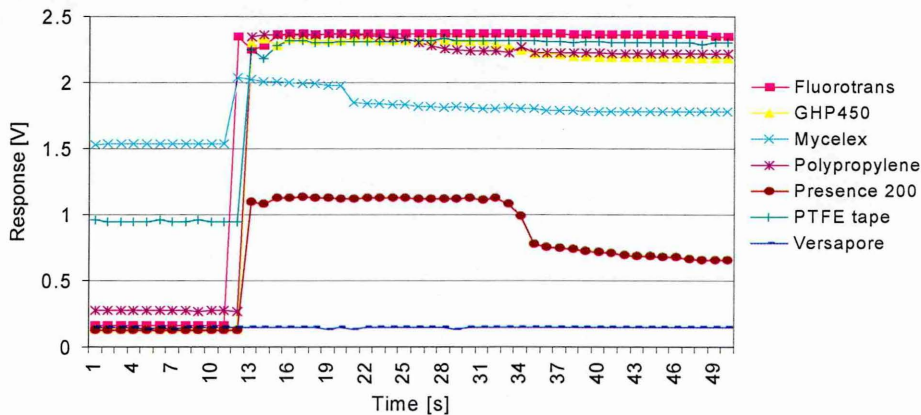


Fig. 4.6 Membrane response to diesel contamination

Membranes (20 mm dia.) were fixed over the glass sensing window of a Mk 3 sensor (3.2.3). After a ~ 10 s baseline period, 16 μ l diesel was pipetted onto the membrane and monitored for ~ 50 s.

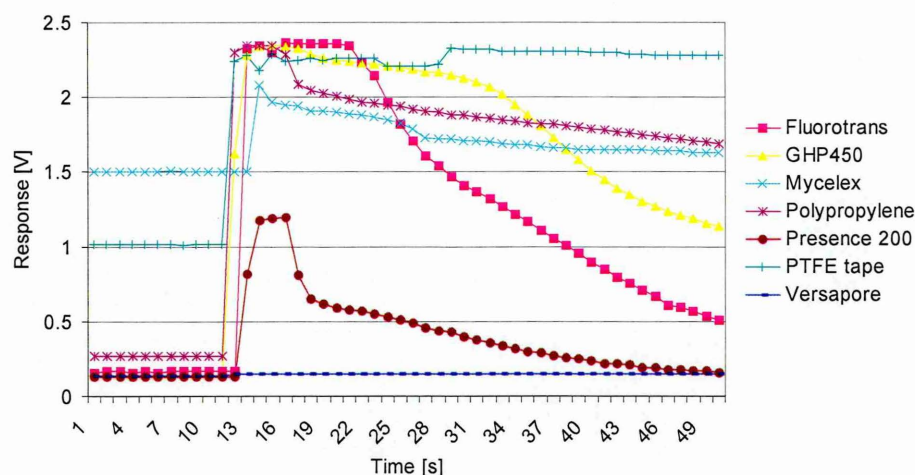


Fig. 4.7 Membrane response to unleaded petrol contamination

Membranes (20 mm dia.) were fixed over the glass sensing window of a Mk 3 sensor (3.2.3). After a ~ 10 s baseline period, 16 μl unleaded petrol was pipetted onto the membrane and monitored for ~ 50 s.

4.2.3.2 Membrane responses to repeat contamination and regeneration through fuel evaporation to air

Membranes with the highest signal responses to all three fuels were: Fluorotrans, polypropylene and PTFE. Tests for membrane regeneration (reversibility of response) were thus conducted only on these three membranes. The possibility of fuel differentiation was also examined by evaluating the signal kinetics of each membrane in response to the two primary types of fuel, unleaded petrol and diesel.

Fig. 4.8 shows that Fluorotrans demonstrated the quickest evaporation/recovery time when contaminated with unleaded petrol and left to evaporate in air. The signal returned to the baseline value of 0.3 V in ~ 3.2 min after each subsequent contamination. Post-diesel contamination, only the Fluorotrans membrane gave some indication of regeneration over an extended 35 min monitoring period (Fig. 4.9). Monitoring was continued immediately to determine maximum regeneration by the Fluorotrans membrane.

After ~ 9 hr, Fluorotrans demonstrated almost complete regeneration, returning to a slightly higher baseline of 0.59 V (Fig. 4.10). Polypropylene and PTFE membranes showed no regeneration after 25 hr. The polypropylene noise exhibited earlier on in the experiment was therefore insignificant.

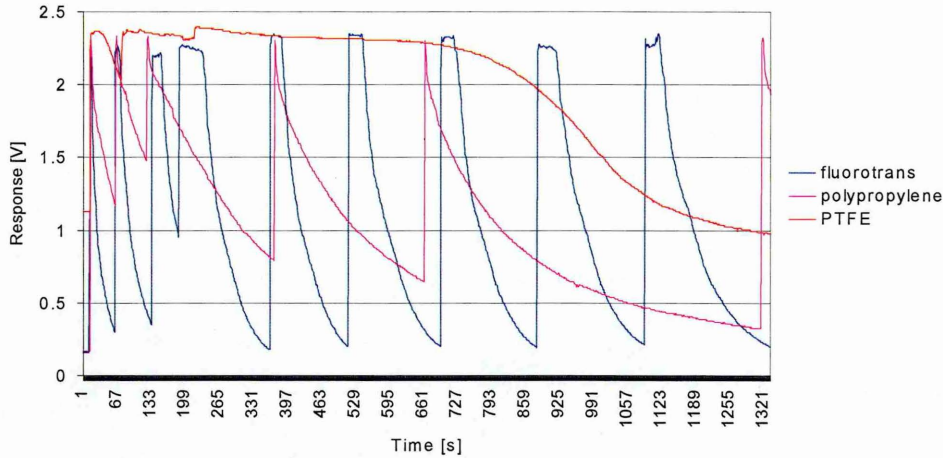


Fig. 4.8 Membrane regeneration in air (petrol contamination)

Four separate aliquots ($8\ \mu\text{l}$) of petrol were placed on the membrane at 60 s intervals (after a 10 s baseline was established). Where membranes had regenerated (signal returned close to original baseline levels, due to volatilisation of component species) further $8\ \mu\text{l}$ aliquots of fuel were added to determine the repeatability of response (3.2.3.1.1).

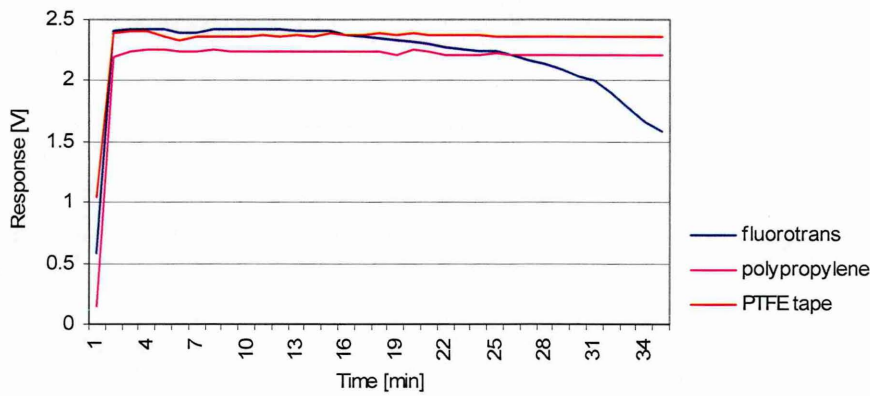


Fig. 4.9 Membrane regeneration in air (diesel contamination)

Four separate aliquots ($8\ \mu\text{l}$) of diesel were placed on the membrane at 60 s intervals (1 s sampling, 3.2.3.1.2). Monitoring was extended to 35 min (5 s sampling). No further aliquots of diesel were added.

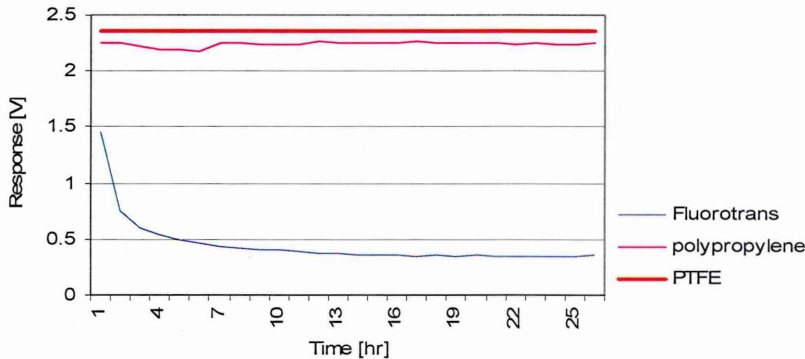


Fig. 4.10 Extended monitoring of membranes in air (after diesel contamination).

As a result of very slow regeneration (Fig. 4.11) monitoring (5 s sampling) was continued over 25 hr.

4.2.3.3 Membrane regeneration by fuel dissolution in water

Regeneration of the membrane within a saturated soil or water matrix was also desirable and so experiments were repeated, with the sensor head and membrane immersed into a stirred water matrix, post-contamination. (3.2.3.2).

The Fluorotrans membrane showed superior regeneration properties after contamination with either petrol or diesel by way of evaporation (Fig. 4.8-4.10) and also by dissolution in water (Fig. 4.11-4.14). After repeated petrol contamination, recovery times through evaporation averaged 3.2 min, whereas regeneration by dissolution in water averaged 12 min. Regeneration in water resulted in a slightly elevated baseline of ~ 0.5 V, which then remained consistent. Polypropylene and PTFE gave no indication of regeneration by dissolution in water.

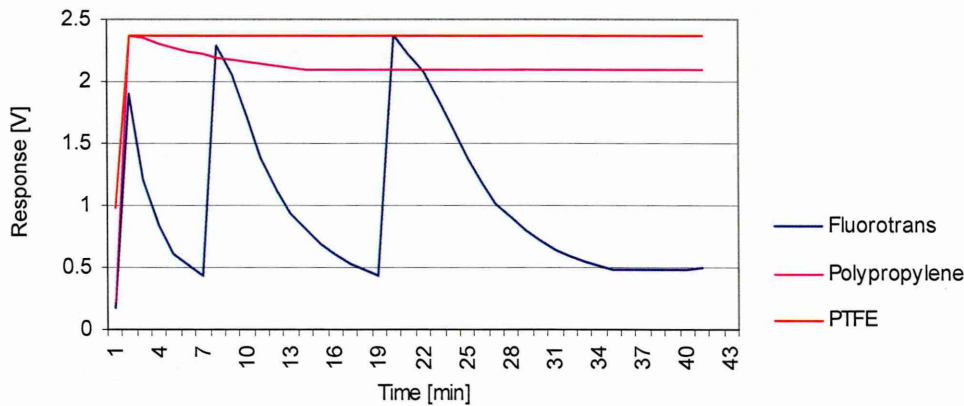


Fig. 4.11 Membrane regeneration in water (after petrol contamination)

An 8 μ l aliquot of petrol was placed onto the membrane and the sensor head immediately submerged in water. Three subsequent aliquots of petrol (8 μ l) were added to each membrane at 60 s intervals. Monitoring (1 s sampling) was initially for 40 minutes. Where regeneration (through dissolution) occurred, further aliquots (8 μ l) were added to determine repeatability of membrane responses (3.2.3.2.1).

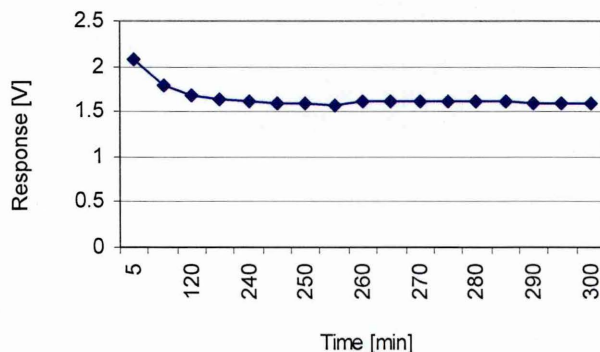


Fig. 4.12 Extended monitoring of polypropylene contaminated with petrol

After contamination of the polypropylene membrane with petrol, monitoring with the sensor submerged in water was continued for a further 5 hr (5 min sampling).

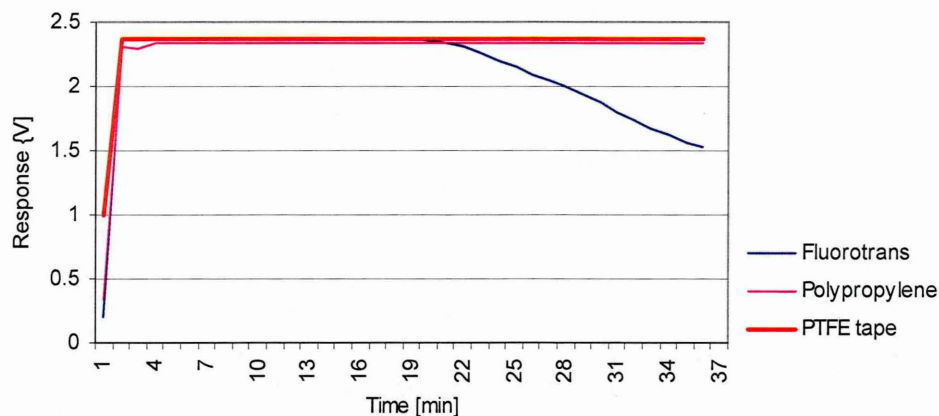


Fig. 4.13 Membrane regeneration in water (diesel contamination)

An 8 μl aliquot of diesel was placed onto the membrane and the sensor head immediately submerged in 400 ml water (stirred) and monitored for 60 s. The sensor was briefly removed, a further 8 μl of diesel added to each membrane and immersed back into the water (3.2.3.2.2).

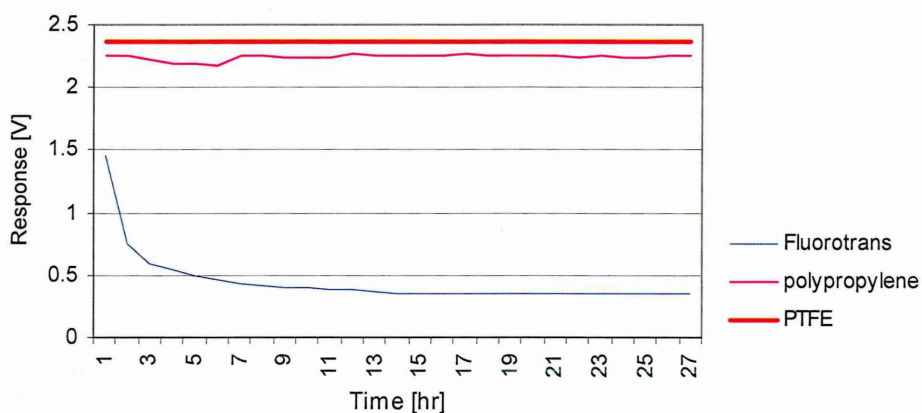


Fig. 4.14 Extended monitoring (diesel contamination)

Following on from Fig. 4.13, monitoring (with the sensor submerged in stirred water, 5 s sampling) was continued for a total of 25 hr.

4.2.3.4 Differentiation between fuel types by response kinetics

The results from 4.2.3.3 (Fig's. 4.11-4.14) show that it was therefore possible to distinguish between the two fuel types: diesel and unleaded petrol by monitoring the signal kinetics from the contaminated membrane. Petrol and diesel will migrate from the Fluorotrans membrane in both air and water matrices. Thus, the rate of migration can distinguish between the two fuels, with retention time of the diesel being much higher than that of petrol. This is attributed to the more volatile and water-soluble components present in petrol (oxygenates), which are not present in the heavier diesel fuel. However, evaporation is a much more efficient means of regeneration/removal of contaminants than by dissolution in water.

4.2.4 Laboratory evaluations of multi-channelled sub-surface sensors (UST simulations)

This series of experiments simulated a leaking underground storage tank. Experiments were conducted under controlled conditions using two Mk 2 sensors and seven Mk 3 sensors, unless otherwise stated. With the exception of 4.2.4.1, 100 ml volumes of either petrol or diesel was deliberately leaked from a sub-surface vessel ('UST') into a tank filled with sand, which was detected by sensors placed within the sand matrix.

4.2.4.1 Preliminary determination of sensor depth positioning in wet sand

Fig. 4.15 shows that sensors triggered according to their distance from the leak point, achieving >1.9 V responses to 200 μ l diesel (Table 4.3). Only sensor 6 showed no response to fuel and indeed had not been exposed, a fact confirmed by an opaque membrane (Fig. 4.16). All triggered sensors had complete membrane translucency.

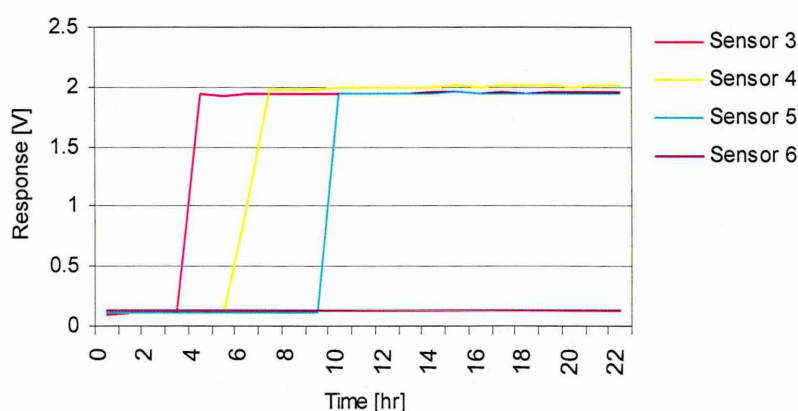


Fig. 4.15 Effect on sensor response of depth and distance from the leak point source.

Four Mk 3 sensors were placed vertically at varying depth and distance from the leak point (3.2.4.2). Sensors were numbered sequentially according to their distance from the leak point (3 was closest). Over 24 hr, 200 ml diesel was leaked into wet sand. Sampling was hourly.

Table 4.3 Sensor response times and signal voltages

Details of the triggered sensor signals (3, 4 & 5) are shown in order of response. Sensor 6 showed no change from it's baseline signal (0.11 V).

Sensor No.	Baseline signals (V)	Initial response		Final signal		Time difference (min)	Depth (cm)	Distance (cm)
		Time (min)	Signal (V)	Time (min)	Signal (V)			
3	0.11	225	1.95	332	1.96	107	6	1
4	0.11	332	1.99	570	2.0	237	7.5	2
5	0.11	571	1.96	571	1.96	239	7.5	6



Fig. 4.16 Post-experimental examination of sensor heads

The four Mk 3 sensors (used in 4.2.4.1) are shown in order (left to right); 6,5,4,3. Sensor heads have been removed to expose the membranes. Translucent membranes indicate exposure to fuel.

4.2.4.2 Determination of circular plume movement around a UST in wet sand

In this experiment, sensors were placed in a circular arrangement around the UST, with the sensors positioned vertically (face down). The sensor heads were positioned 1 cm beneath the level of the UST base. The sand had been saturated with water (3 l) prior to commencing the fuel leak (3.2.4.3).

When sensors were placed in a circular formation underneath the UST (Fig. 3.6), sensor 1, situated immediately under the leak point showed a response to 5 ml of leaked fuel after 25 min (Fig. 4.17). Fuel leakage was hindered by water-saturation of the sand. Once the water inflow was stopped (30 min into the experiment, 3.2.4.3.1), responses improved. After 4 hr, 6 sensors showed a positive response indicating the plume had spread under the UST base (Table 4.4). The last sensor to respond (sensor 6) was furthest from and opposite to the leak point. Sensor 2 was the older Mk 2 design and so had a higher baseline signal than the other Mk 3 sensors (Section 2.5.2).

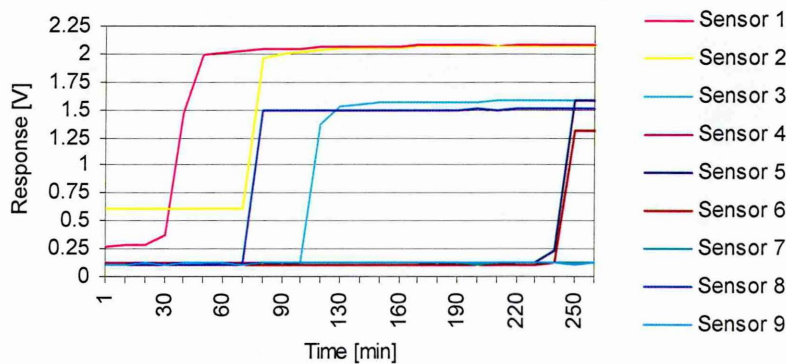


Fig. 4.17 Circular sensor siting beneath the UST in wet sand

Mk 3 sensors were positioned vertically around the UST and pushed 1cm beneath the level of the flask base. Total depth from surface was 3 cm (3.2.4.3). Sensors 1 & 2 were the Mk 2 design. Monitoring was for 4 hr with 100 ml diesel leaked from the UST.

Table 4.4 Sensor response times and signal voltages

The details of the sensors triggered in Fig. 4.17 are shown in order of response.

Sensor No.	Baseline signals (V)	Initial response		Final signal		Time difference (min)
		Time (min)	Signal (V)	Time (min)	Signal (V)	
1	0.29	25	1.99	75	2.306	50
2	0.62	73	0.63	81	1.98	8
8	0.11	76	0.56	79	1.51	3
3	0.11	106	0.23	112	1.58	6
5	0.11	225	0.21	238	1.58	13
6	0.11	229	0.22	239	1.31	10

4.2.4.3 Determination of circular plume movement around a UST in dry sand

Heavily water-saturated sand (as used in the previous experiment, 3.2.4.2) had hindered fuel leakage from the flask. Consequently, in these experiments, dry sand was used with two water inflow rates - 4 and 8 ml/min. After 2 hr, it was visually observed that all diesel (100 ml) had leaked out of the flask (into the surrounding sand). In both experiments, the two sensors positioned either side of the leak point had triggered within 25 min. (Fig. 4.18). No further sensors responded during the extended 48 hr monitoring.

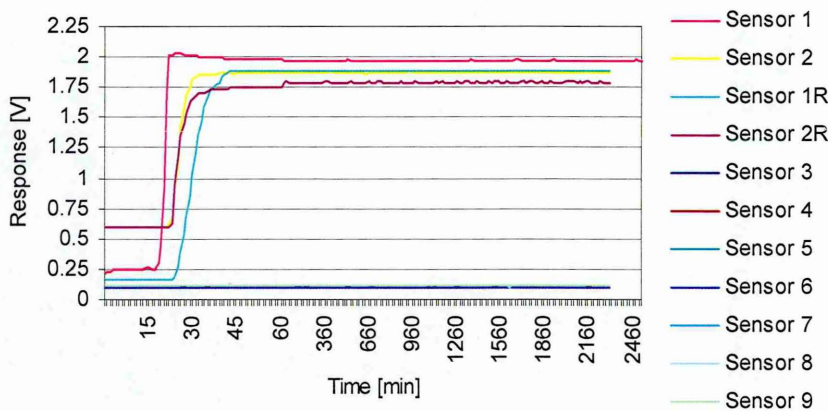


Fig. 4.18 Diesel plume movement around a UST in dry sand; sensors in a circular formation.

Two sets of experimental results are shown (3.2.4.3.2). Sensors 1 & 2 were of the Mk 2 design, all remaining sensors were Mk 3. Repeat results have been indicated 'R' in the legend key. No changes in sensor signals were observed after 48 hr monitoring.

Fig. 4.18 shows the sensor responses to diesel when in a circular arrangement in dry sand. In this case, both sensors placed either side of the leak point triggered within ~300 s of each other (3.2.4.3.2). These signals remained consistently high throughout the 48 hr monitoring (Table 4.5). No other sensors responded and later inspection confirmed that these had not been exposed to fuel. The diesel had leaked out and rapidly triggered sensor 1, immediately in front of the leak point and had then moved to the right of the UST, triggering sensor 2. The diesel plume

must have then moved downwards through the sand, as sensor 9, placed 3 cm behind sensor 1 was not triggered. Theoretically, the plume would then have moved towards the left of the tank, driven by the water movement/water extraction (3.2.4.3).

Table 4.5 Combined sensor response times and signal voltages

Only the triggered sensor values (from both experiments, 4.2.4.3) are shown as the other sensors did not respond. The repeat results are indicated by the letter R. Sensors 1 & 2 were positioned either side of the leak point at equal depths.

Sensor No.	Baseline signals (V)	Response	
		Time (min)	Signal (V)
1	0.24	17	2.04
2	0.60	22	1.87
1R	0.17	24	1.89
2R	0.59	23	1.76

4.2.4.4 Determination of vertical diesel plume movement with a horizontal fan of sensors

When sensors were laid horizontally in a fan formation and at three sub-surface depths (3.2.4.4), it was possible to trace the vertical flow of diesel (Fig. 4.19). All three surface sensors situated around the leak point responded rapidly (within ~80 s, Table 4.6). After ~1 hour, a mid-depth sensor (6) responded. After a further 25 min, a single base sensor triggered (3). A steady decrease in the signal from sensor 8 (placed level with the leak point) occurred throughout – suggesting removal of fuel from the membrane surface. The response from base sensor (3) correspondingly increased, indicating increasing fuel concentrations immediately above the gravel layer, yet held within the sand.

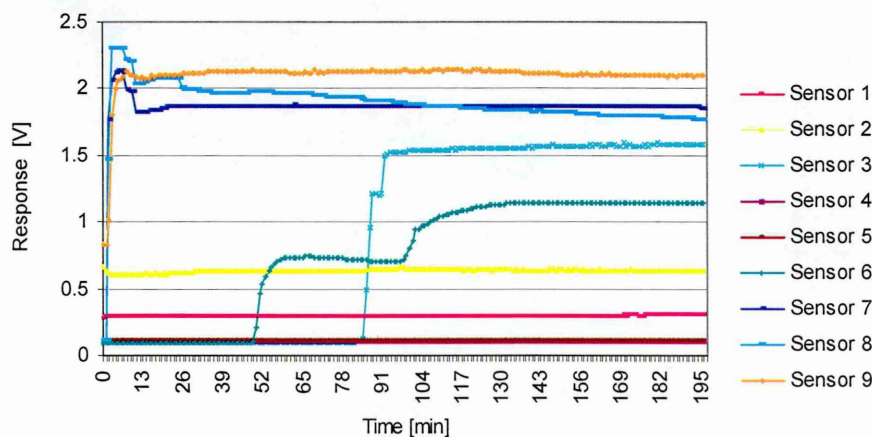


Fig. 4.19 Vertical diesel plume movement in damp sand

Sensors were positioned horizontally in groups of three (in a fan formation) at three different depths in damp sand (3.2.4.4). The experiment was run for 3 hr with 100 ml diesel leaked from the UST.

These results indicate that the fuel plume spread horizontally outwards from the UST leak point. The plume preferentially flowed to one side (left) and then travelled vertically down through the sand horizon. The bias of plume flow indicates the heterogeneity of an apparently homogeneous microenvironment.

Table 4.6 Sensor responses at variable depths (fan formation)
The details are shown in order of response.

Sensor level	Sensor no.	Baseline signals (V)	Response time	Max. signal (V)
Surface	9	0.11	10 s	2.13
Surface	8	0.12	110 s	1.98
Surface	7	0.11	90 s	1.86
Mid-depth	6	0.1	46 min	0.73/1.14
Base	3	0.1	82 min	1.54

4.2.4.5 Determination of diesel plume movement with a vertical grid of sensors

Fig. 4.20 shows that horizontal plume movement could be tracked with the sensors placed at depth and in a grid formation (3.2.4.5). All 100 ml diesel had leaked from the UST after 15 min. The bulk mass of diesel must have moved vertically as rapid responses (≥ 10 min) were obtained from the first row of sensors (Table 4.7). The horizontal plume movement was successfully tracked by the remaining sensors. These show a clear correlation between response times and distance from the point source of contamination.

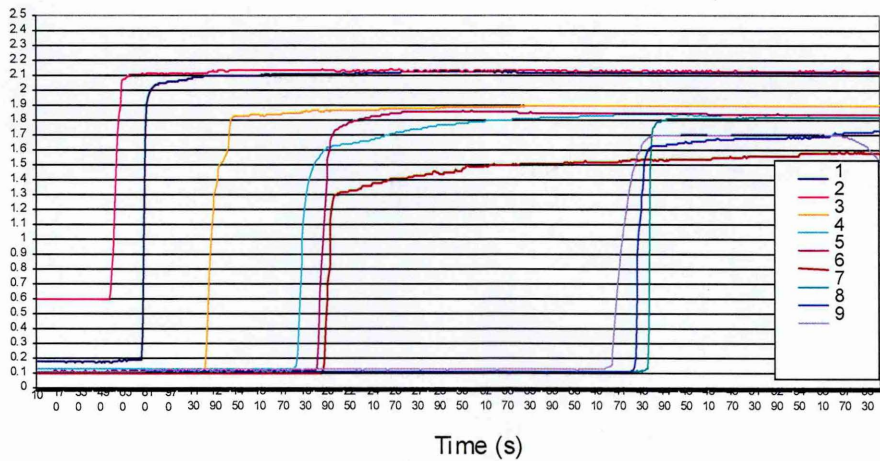


Fig. 4.20 Horizontal plume movement of 100 ml diesel in damp sand.
Sensors were placed uniformly in a grid formation vertically into damp sand (3.2.4.5). The sensor heads were 1 cm above the gravel base layer. The base of the UST vessel was 7 cm above the sensors. A 100 ml volume of diesel was leaked. Monitoring was for 2 hr.
** This graph could not be formatted due to a corrupted data file*

Table 4.7 Sensor responses in a grid formation to 100 ml diesel
Signals from triggered sensors in Fig. 4.20 are shown in order of sensor response.

Sensor	Baseline (V)	Initial response time (min)	Maximum signal (V)	Total signal Increase (V)
2	0.6	8	2.13	1.53
1	0.18	12	2.13	1.95
3	0.1	30	1.9	1.8
4	0.13	31	1.7	1.57
5	0.11	33	1.8	1.69
6	0.1	34	1.59	1.49
9	0.11	67	1.7	1.59
8	0.11	70	1.72	1.61
7	0.11	72	1.8	1.69

4.2.4.6 Determination of petrol plume movement with a vertical grid of sensors

The determination of plume direction was repeated with petrol contamination (3.2.4.5). The order of responses again corresponded to sensor distance from source (Fig. 4.21). The first row of sensors (sensors 1, 2 & 3) placed in line underneath the leak point (Fig. 3.8) responded within 15 min. The second row of sensors (sensors 3, 4 & 5) placed 4 cm away from the previous row of sensors responded ~ 15 min later. The final row of sensors (6, 7 & 8) sited 8 cm back from the leak point all responded after ~ 1 hour (Table 4.8). It was also possible to see variations in the plume spread as it vertically flowed down through the sand horizon, indicated by the order of sensor response.

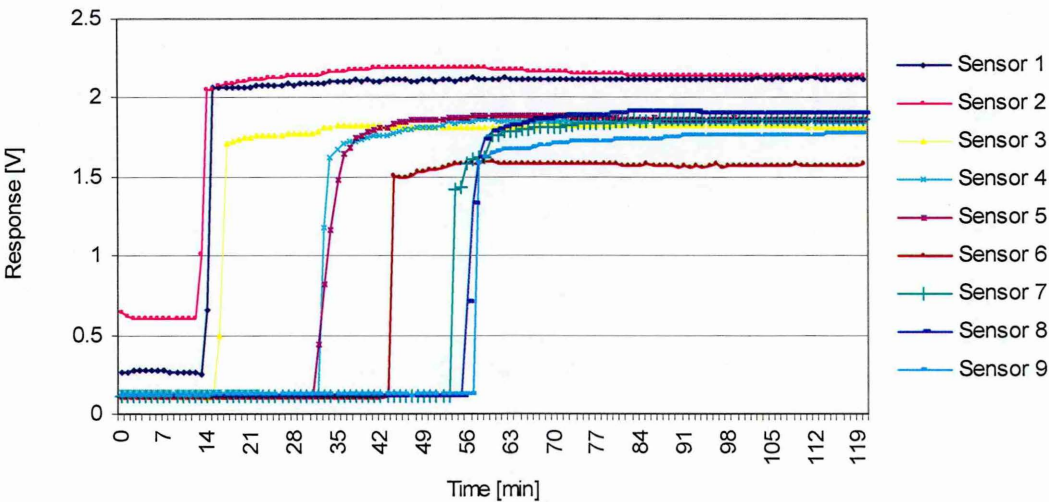


Fig. 4.21 Sensor responses in a grid formation to 100 ml petrol
Sensors were again placed in a grid formation (3.2.4.5) in damp sand (Fig. 3.8). In this experiment, 100 ml petrol was leaked, monitoring was for 2 hr.

Table 4.8 Sensor responses in a grid formation to 100 ml unleaded petrol in dry sand.
Triggered sensor responses (as shown in Fig. 4.21) are shown in order of response.

Sensor	Initial response time (min)	Baseline (V)	Maximum signal (V)	Total signal Increase (V)
2	13	0.65	2.2	1.55
1	14	0.27	2.15	1.88
3	15	0.11	1.83	1.72
5	33	0.11	1.89	1.78
4	34	0.11	1.87	1.76
6	44	0.1	1.61	1.51
7	54	0.11	1.85	1.74
8	56	0.12	1.92	1.8
9	58	0.11	1.81	1.70

4.2.4.7 Determination of membrane regeneration through dissolution by soil water movement

In this experiment, the sensors were again placed vertically face down in damp sand with the sensor heads 1 cm above the gravel base in a circular fashion (Fig. 3.9). A 100 ml volume of diesel was leaked and was detected by 8 out of 9 sensors (Table 4.9). Monitoring was continued for 17 hr to determine if the diesel contaminant would eventually be removed from the sensor membrane by natural dissolution through the continuous passage of water (Fig. 4.23).

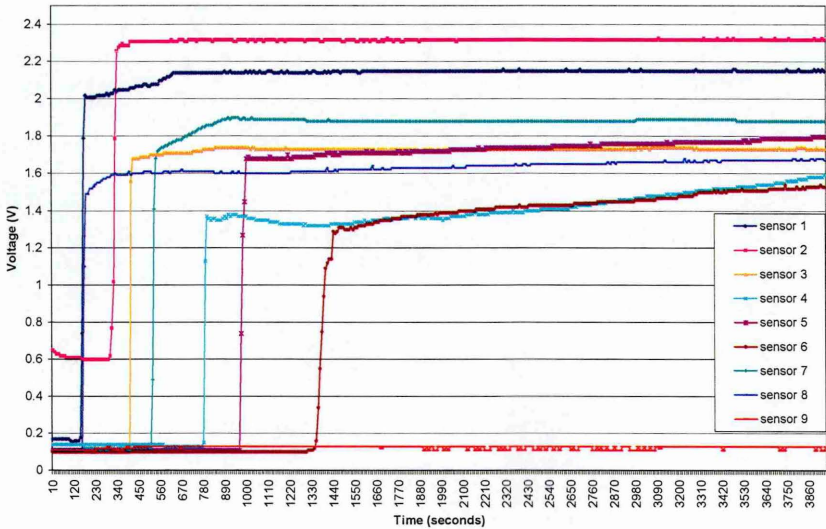


Fig. 4.22 Determination of sensor response (circular formation) and membrane regeneration by soil water movement.

Sensors were placed vertically into damp sand (pre-wetted with 3 l water) with the sensor heads 1 cm above the gravel base layer in a circular fashion around the UST. The base of the UST vessel was 7 cm above the sensors (3.2.4.6). A water inflow of 8 ml/min with water extraction pumped at 10 rpm to mimic groundwater flow. After 100 ml diesel had leaked, monitoring was extended for 17 hr to determine potential regeneration *in situ*.

Fig. 4.22 shows that very rapid responses occurred when sensors were again placed at depth. In this instance, a circular formation around the UST was used (3.2.4.6). All sensors responded. Sensors 4, 5 and 6 showed the least increase in signal. These sensors were closest to the water inflow and so the lowered response was indicative of fuel dispersion driven by water movement. When monitoring was extended over a further 17-hour period, these three sensors regenerated, all returning to baseline signals, most probably through dissolution by water in soil (Fig. 4.23). Regeneration of fuelled membranes submerged in water has been previously demonstrated (4.2.3.3).

Table 4.9 Response times of sensors to 100 ml diesel in damp sand
The sensors are numbered according to their chronological response to 100 ml diesel.

Sensor	Baseline (V)	Initial response time (min)	Initial signal response (V)	Maximum signal (V)
1	0.17	2	2.02	2.15
8	0.11	3	1.49	1.67
2	0.65	5	2.29	2.32
3	0.10	6	1.68	1.73
7	0.11	8	1.73	1.80
4	0.14	13	1.37	1.59
5	0.11	16	1.68	1.80
6	0.10	22	1.29	1.53
9	0.11	NR	NR	NR

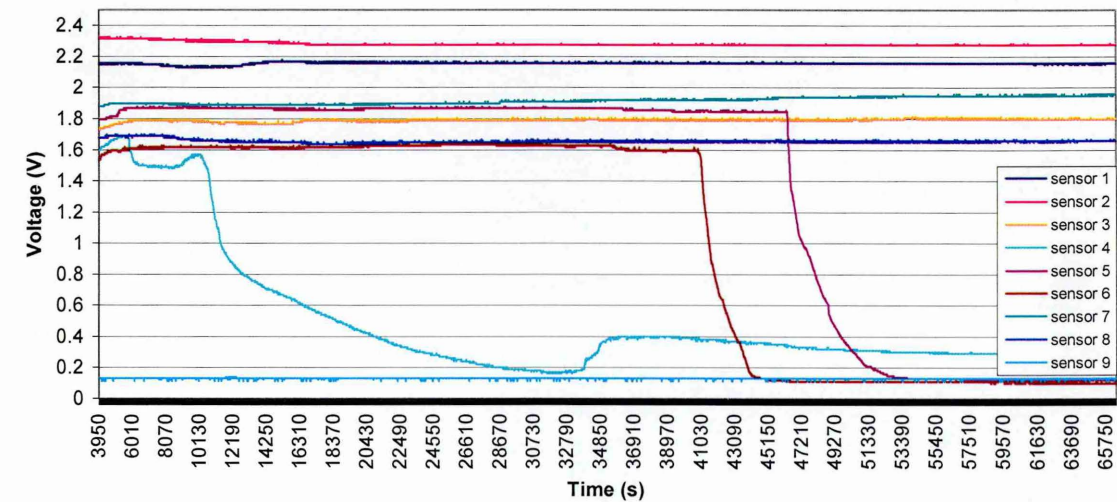


Fig. 4.23 Extended monitoring to determine membrane regeneration *in situ*
Extended monitoring (17 hr) after sensors had triggered (Fig. 4.22, Table 4.9). Membrane regeneration post-diesel contamination was demonstrated by sensors 4, 6 and 5 within 17 hr.

4.2.5 Quantitative evaluation of the Fluorotrans membrane/Mk 3 sensors in response to fuels in different soil types and conditions

Whilst the UST simulations in 4.2.4 demonstrated the sensors ability to detect even very small fuel spills (≥ 20 ml), it was of particular interest to determine how much fuel could elicit a significant response and what were the effects of different soil substrates. Heavy soil water content had impeded fuel plume movement (4.2.4.2) and earlier experiments had previously shown that water could seriously impede fuel absorption by the membrane (4.2.2).

The following series of experiments (3.2.5) addressed these factors by placing Mk 3 sensors into homogenised soil/water mixtures contaminated with different fuels: Unleaded petrol, diesel, central heating oil and MTBE.

Fig. 4.24 shows that the most rapid and highest responses to all fuels were exhibited in totally dry soils. All fuels tested were detectable at small contamination volumes of 400 μ l in 5 g of dry soils. Fuel generally took longer to absorb onto the membrane (and induce a sensor response) in heavy clay soil. MTBE failed to initiate a significant sensor response in dry clay soil. Experiments were then repeated (3.2.5) using wet (50% water) and totally saturated soils (Figs. 4.25-4.26).

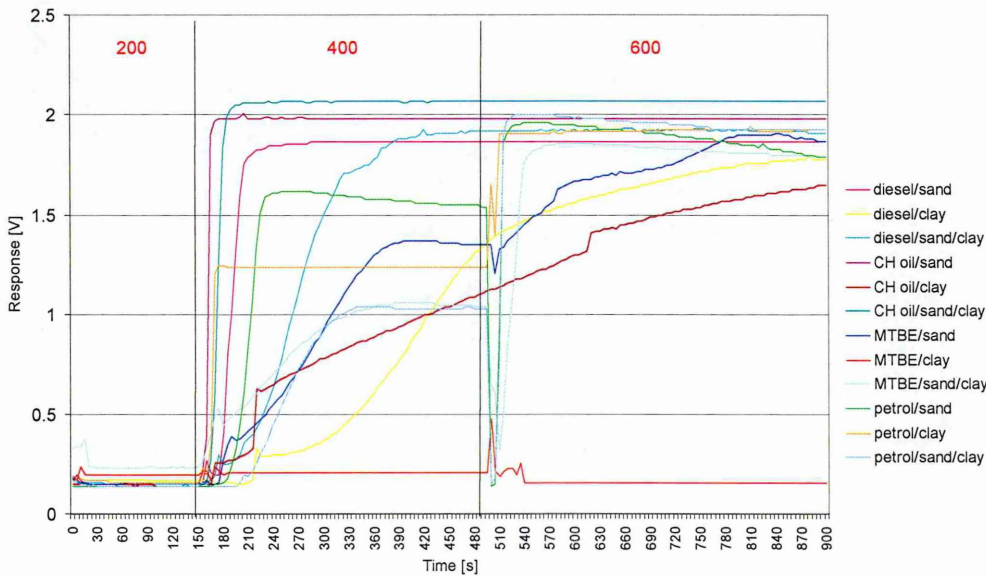


Fig. 4.24 Sensor response to fuels in dry soils (kiln-dried)

Mk 3 sensors were tested in mixed soil/fuel combinations (3.2.5). These are indicated in the legend key. The volume of fuel (μ l) added to soil samples (5 g) is shown.

Fig. 4.25 shows fuel detection in wet soil. The best sensor responses to all fuels were in a sand/clay mixture. Fig. 4.26 (fuel detection in fully-saturated soil) strongly suggests/corroborates the previous finding that water indeed impedes the contamination of the membrane by hydrophobic fuels. Sand produced the best responses in water-saturated conditions. In this case, the more polar MTBE gave the highest signal.

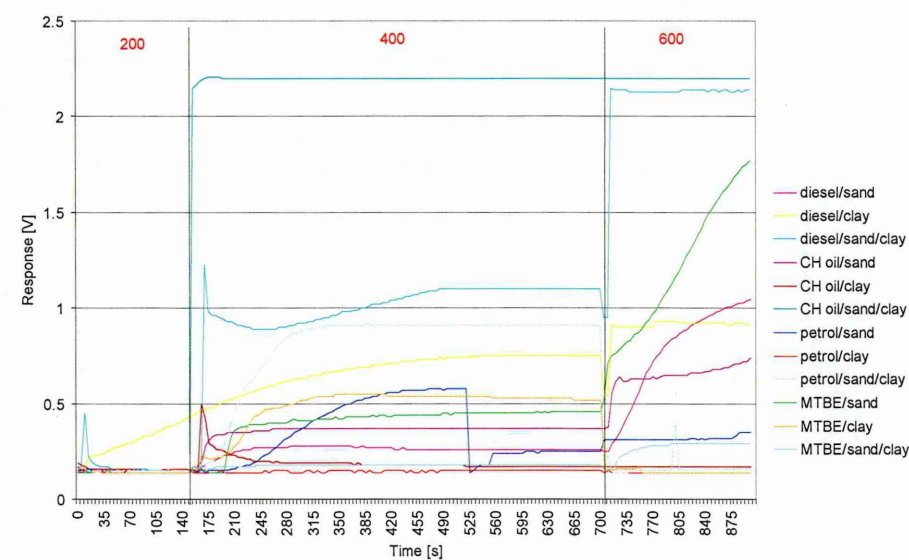


Fig. 4.25 Sensor response to fuels in wet soils
Mk 3 sensors were tested in pre-mixed soil/fuel combinations (3.2.5) as indicated in the key. The volume of fuel (μl) added to soil is shown. Water content was 50%.

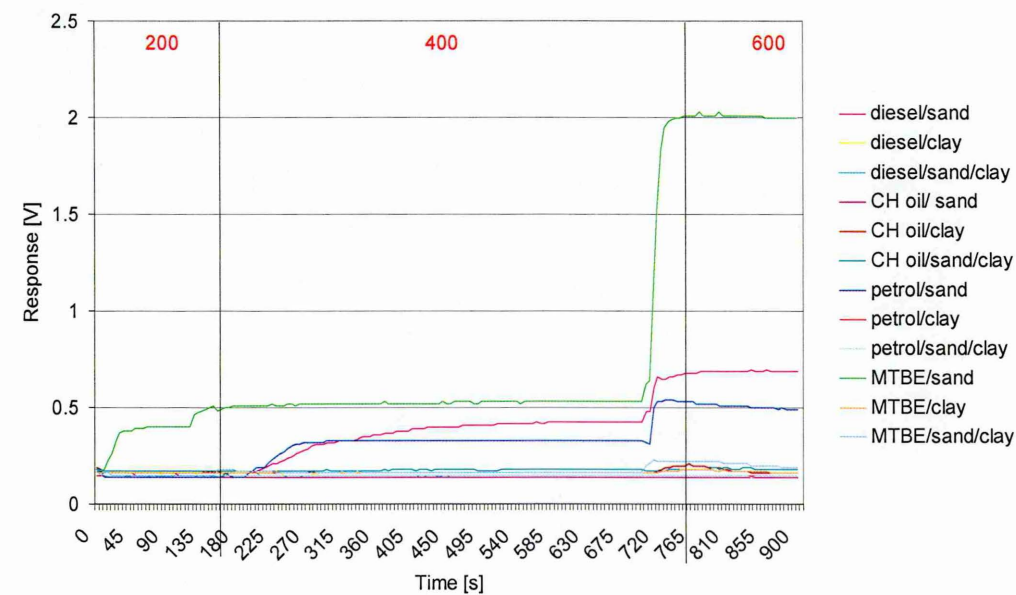


Fig. 4.26 Sensor response to fuels in water-saturated soils
Mk 3 sensors were tested in pre-mixed soil/fuel combinations (3.2.5), as indicated in the key. The volume of fuel (μl) added to soil is shown.

4.2.6 Membrane Resistance to Biofouling

The ability of the Fluorotrans membrane to withstand either colonisation with biofilms or other biological degradation was of primary importance. Such resistance would be vital to preserve the membrane integrity during long-term sub-surface use and consequently the overall sensor performance. Both induced biofouling and natural biofouling through groundwater exposure were examined (3.2.6).

4.2.6.1 Induced membrane biofouling

No obvious biofouling occurred on membranes incubated with *P. fragi*, *S. cerevisiae* and *P. verrucosum*. All these membranes responded normally to fuel contamination and total translucency was obtained with 8 μ l diesel (Fig. 4.27).

Fig. 4.28 shows a single membrane replicate colonised by *A. ochraceus*. The organism appeared to have attached to the membrane on the outer edges and colonised over one surface only. The growth was strong enough to cause the membrane to curl. The remaining two replicates showed no colonisation. Whilst uncolonised membranes responded to 4 μ l diesel, 8 μ l was required to cause a response on the heavily colonised surface. At $\times 200$ magnification, fungal hyphae could be seen at the outer edge of the membrane (Fig. 4.29).

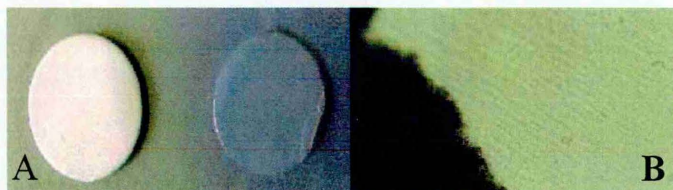


Fig. 4.27 Membrane response to fuel after exposure to *P. fragi*.

A) Unfuelled (opaque) membrane and a fuelled (translucent) membrane (8 μ l diesel); B) Translucent membrane ($\times 200$ magnification). The dark region is membrane reverting back to opacity due to fuel evaporation.

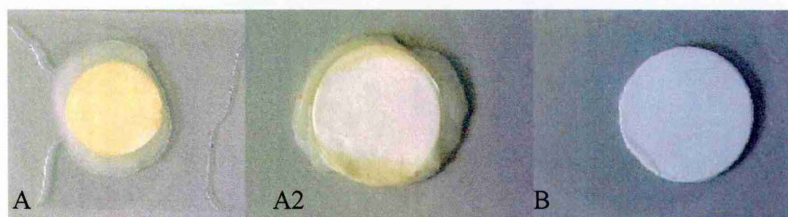


Fig. 4.28 Membrane colonisation of a single replicate by *A. ochraceus*

A) and A2) Two sides of same membrane, exposed for 14 days in nutrient broth suspension (3.2.6.1.2); B) Uncolonised membrane (post exposure to *A. ochraceus*).

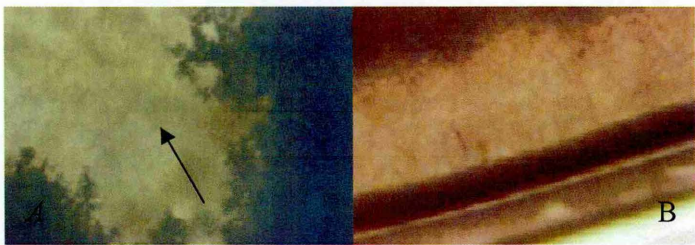


Fig. 4.29 A) *A. ochraceus* colonised membrane (underside x 100 magnification). The membrane still responded to diesel (8 μ l), shown by the lighter, translucent regions (arrowed). The darker regions show the membrane reverting to opacity as a result of fuel evaporation. B) Fungal hyphae on outer edge of membrane (x 200 magnification).

Fig. 4.30 shows growth of the fungus *F. poae* had established on the roughened membrane edge in one replicate. The membrane had stained red, yet still turned translucent with 8 μ l diesel. The surface membrane structure and response to fuel did not appear to be compromised by the presence of hyphae (Fig. 4.31) and so it was concluded that biofilms did not generally colonise the membrane in laboratory trials and would not therefore reduce membrane sensitivity to fuels.



Fig. 4.30 Membrane post-inoculation with the crop fungus *F. poae*. Extensive growth on one replicate is shown (left). All 3 membranes turned translucent on exposure to diesel (8 μ l) regardless of the degree of colonisation or discoloration (as seen right - 3).

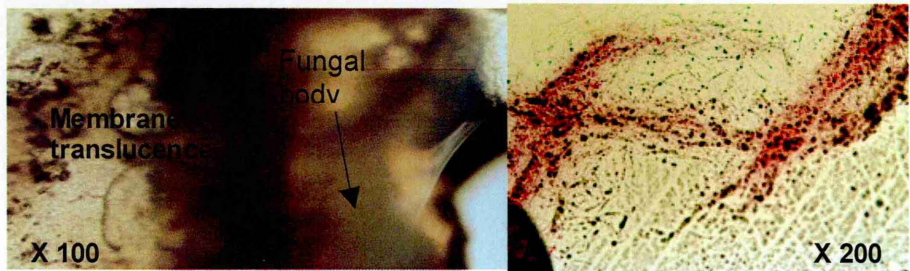


Fig. 4.31 *F. Poae* colonisation of 1 membrane replicate. Membrane translucence (induced by 16 μ l fuel) can be seen underneath the fungal colony (x 100). Under x 200 magnification, hyphal attachment to the translucent membrane surface is apparent.

4.2.6.2 Membrane biofouling under natural ground conditions (factorial experiment)

The Fluorotrans membrane was resistant to natural biofouling by groundwater (3.2.6.2). No discoloration, colonisation or degradation was evident when examined by light microscopy (Fig. 4.32). Groundwater-exposed membranes responded with the same degree of translucency and had the same fuel retention time (4 μ l diesel) as new membrane.

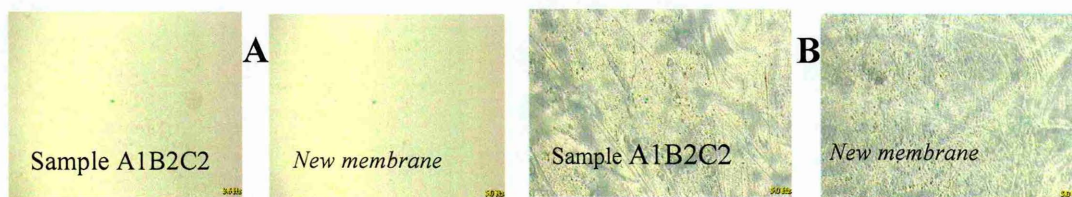


Fig. 4.32 Membrane resistance to natural biofouling.

Membranes had been exposed to natural groundwater for 6 months (3.2.6.2). A representative sample A1B2C2 is shown compared to a fresh membrane (not exposed to fuel). A) Opaque state; B) Membranes exposed to 4 μ l diesel (all under $\times 10$ light magnification).

Because there were no measurable adverse effects on the membranes tested, further factorial analysis was precluded. The Fluorotrans membranes were therefore considered sufficiently resistant to natural biofouling by groundwater, under the conditions employed.

4.2.7 Extraction of BTEX compounds from fuel-contaminated membranes

Experiments were conducted to see if fuel contaminants could be extracted from the Fluorotrans membrane and subsequently analysed by immunoassay. As previously mentioned in Chapter 1, if petrol and diesel can be differentiated as a result of the BTEX content, the sensors may be considered a semi-analytical tool, which could indicate the most likely contaminant and, in the case of a multi-UST site, such as a petrol station forecourt, pinpoint the leaking UST. Analysis was made using the SDI RaPID Total BTEX immunoassay kit (1.5.2.2). Diesel-spiked membranes and diesel-contaminated membranes from a field trial (3.3.3) were assessed (3.2.7).

Fig. 4.33 shows that extraction with 1% MeOH was marginally better for removing both petrol and diesel from the Fluorotrans membrane. A 50% dilution of the MeOH extract resulted in $\sim 50\%$ decrease in BTEX concentration. Dilution of the water-based extract appeared to lose some BTEX content, most probably non-dissolvable compounds, which would not have mixed with the water.

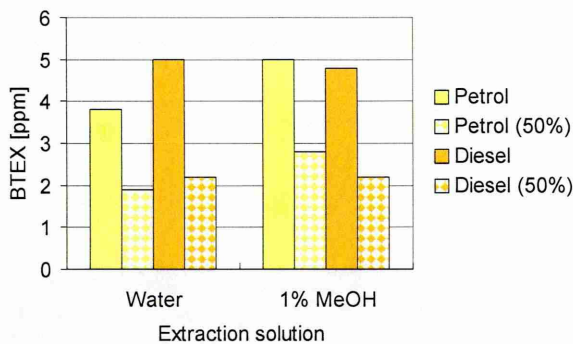


Fig. 4.33 Determination of BTEX after membrane extraction

Extractions of fuels were made with RO water and 1% MeOH solutions (3.2.7). Extract dilutions (50%) were made with RO water (n=1). Analysis was performed using the SDI RaPID Total BTEX immunoassay (1.5.3).

Fig. 4.34 shows that a high BTEX content was extracted from some of the field trial sensor membranes, which corresponded to the visual degree of translucency and the response obtained to the initial contamination *in-situ* (4.3.2.2). The membrane from sensor 3 (untriggered) yielded only a small (100 ppb) quantity of BTEX.

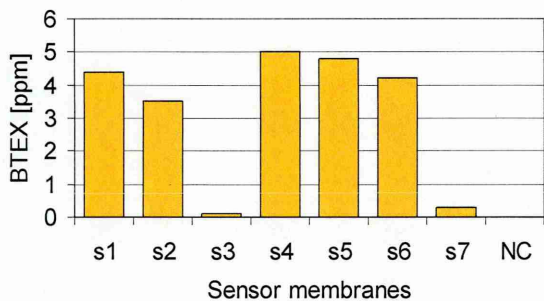


Fig. 4.34 Extraction of BTEX from membranes contaminated in a field trial.

Membranes had been contaminated with diesel during a lysimeter field trial (3.3.3). Membranes were removed from excavated sensors and placed into 1% MeOH (3.2.7.1). NC, fresh membrane control.

4.2.8 Evaluation of plastic materials for Mk 4 sensing windows

A range of plastics were evaluated as replacement materials for the glass sensing window (covering the NIR diode) on the sensor body, since repeated use in field trials had found that the glass deteriorated around the edge as a result of repeated tightening of the sensor head. Evaluation consisted of exposure to unleaded petrol and diesel (3.2.8). PTEG was chosen due to its chemical resilience and infrared transparency. Polycarbonate, Polystyrene and Perspex were also tested.

Fig. 4.35 shows that polystyrene and polycarbonate plastics were both adversely affected by free-phase fuel contact. Overall, unleaded petrol had a much more severe effect. This was most likely due to the elevated ether content, as MTBE and alternative ethers adversely affected the polycarbonate tubes used for preparation of high ($\geq 10,000$ ppm) concentration solutions for analysis with the MTBE immunoassay (Chapter 6). Conversely, PTEG appeared resistant to chemical degradation by both fuels (Fig. 4.36). The red dye (Texas red) present in the agricultural diesel had stained the Perspex and could not be removed. Therefore, only PTEG could potentially be suitable as a replacement material for the glass sensing window of the sensor body (housing the NIR diodes), although further evaluations would be necessary. However, due to later modifications with the Mk 4 sensor design (Section 2.5.4), the problem of glass damage (though overtightening of the sensor head) was overcome. Glass was subsequently retained as the material of choice.

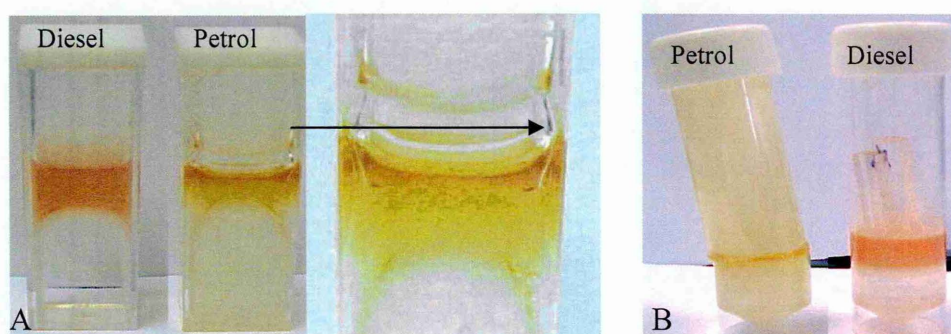


Fig. 4.35 Polystyrene & Polycarbonate after free-phase fuel exposure

Degradation of polystyrene cuvettes (A) and polycarbonate tubes (B) by free-phase fuels. Both were filled 1:2 (v/v) with fuel/water, shaken, sealed and left for 24 hr (3.2.8.1).

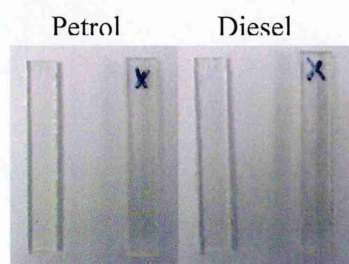


Fig. 4.36 PTEG & Perspex after free-phase fuel exposure

PTEG and Perspex strips were placed into 1:2 (v/v) mixtures of fuel/water (resulting in 50% coverage) and left for 24 hr (3.2.8.2). Exposed strips are marked 'X' and compared to an unexposed strip.

4.3 FIELD-BASED MONITORING TRIALS

4.3.1 Preliminary field monitoring using two sub-surface sensor designs for determining petrol contamination

The aim of this preliminary field monitoring experiment was to ascertain how the Medsci data logging unit (DLU, Section 2.6.2) and linked sub-surface sensors performed in an external environment for a prolonged period of time. Sub-surface sensors (a prototype 'Drallim' sensor and two MK 1 sensors) were placed in a sand-filled tank and a fuel leak initiated (3.3.2).

4.3.1.1 Data acquisition and relay

The Medsci DLU accumulated multi-channel data sets from the sensors and successfully relayed data to multiple email recipients via daily SMS emails. Each emailed data set was successfully converted from the extracted binary (*.dat) files (using the IlogExtract.exe programme (2.6.2.2) and imported into Microsoft Excel for analysis. The monitoring system was successfully reprogrammed with a standard laptop computer *in situ*.

4.3.1.2 Sensor signal responses

The Mk 1 sensors had lower baseline signals compared to the Drallim prototype. Small cyclic baseline fluctuations were exhibited by all 3 sensors, which were due to environmental temperature changes. No sensors responded to repeated petrol contamination (Table 3.8, Section 3.3.2). No cyclic fluctuations were evident when the same sensors were monitored by the Medsci DLU under controlled, laboratory conditions (3.3.2.1).

These preliminary results contributed to the redesign and redevelopment of both the Medsci DLU and the sub-surface sensors (Mk 2 design) and are discussed fully in Chapter 2 (2.5.1).

4.3.2 Lysimeter-based field monitoring trials

Long-term monitoring trials were carried out with an array of the redesigned Mk 3 sub-surface sensors (2.5.2-2.5.3). A lysimeter was obtained specifically for conducting the outdoor field monitoring trials (Fig. 3.12 & 3.13). Four long-term trials were conducted with repeated use of the Mk 3 sub-surface sensors (3.3.3) and the upgraded Medsci DLU unit (2.7).

4.3.2.1 Lysimeter winter trial #1 (diesel in sand)

The winter lysimeter trial #1 (diesel in sand, Fig. 4.37) shows that the sensors responded in accordance to their placement directly around the leak point and their position in relation to vertical phase flow through the lysimeter (Fig. 3.12). Sensors 1 and 2 both responded to the first

leakage of 0.5 l diesel within 15 min. Sensor 3 also responded within 15 min after the 2nd fuel leak (0.5 l). All signals remained stable through weeks 3-4. Post-trial examination of sensors confirmed that those that had triggered all had high membrane translucency.

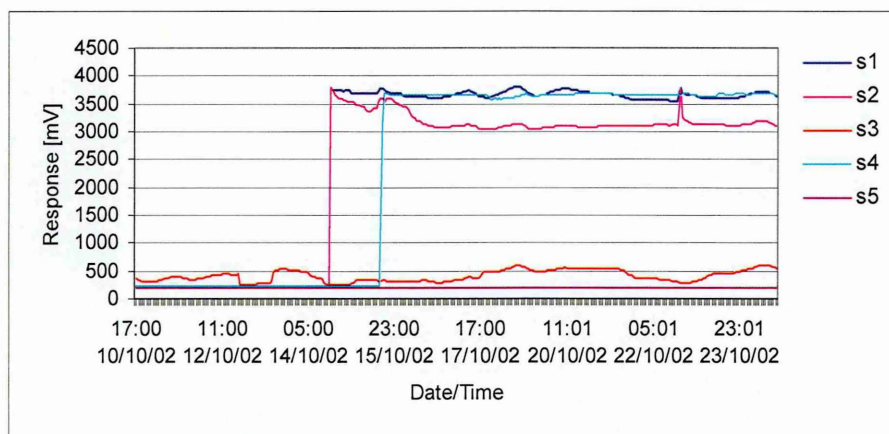


Fig. 4.37 Sensor data from lysimeter winter trial #1 (diesel in sand)

Five Mk 3 sensors were placed around the UST at varying depths (3.3.3). The trial was run for 1 month. A deliberate UST leak was initiated in week 2, with 0.5 l diesel leaked on 14/10/02 with a further 0.5 l leaked 15/10/02 (Table 3.9). Data sampling was hourly. Results from sensor trigger events in weeks 2 and 3 are shown.

In the Spring/Summer lysimeter trial #2 (diesel in sand, Fig. 4.38), sensors 1 and 2, closest to the leak point, triggered within 6 hr of the first fuel addition (0.5 l). The signals of all triggered sensors remained high, regardless of weather conditions (extreme wet to high heat, 29 °C). Loss of sensor signal corresponded to battery failure in week 5. Once this was replaced, signals returned to previous levels. After 12 weeks monitoring, all sensors had triggered except sensor 3 (positioned centrally under the leaking UST) and sensor 7 (base of lysimeter at 1 m depth).

Fig. 4.39 shows rapid sensor response to a single petrol leak (0.5 l) contamination of topsoil. With the exception of sensor 3, placed centrally underneath the UST, all sensors had responded after 8 days. Membrane regeneration, presumably through evaporation and dissolution by natural precipitation, was apparent during this trial. Rapid downward movement of a diesel plume (in topsoil) was also demonstrated in trial 4 (Fig. 4.40). Sensors placed at depths of 10 cm and 20 cm respectively responded within 24 hr of an initial 0.5 l diesel leak. Sensors placed directly next to the leak point responded within 15 min. All sensors had triggered within 4 weeks.

4.3.2.2 Lysimeter Spring/Summer trial #2 (diesel in sand)

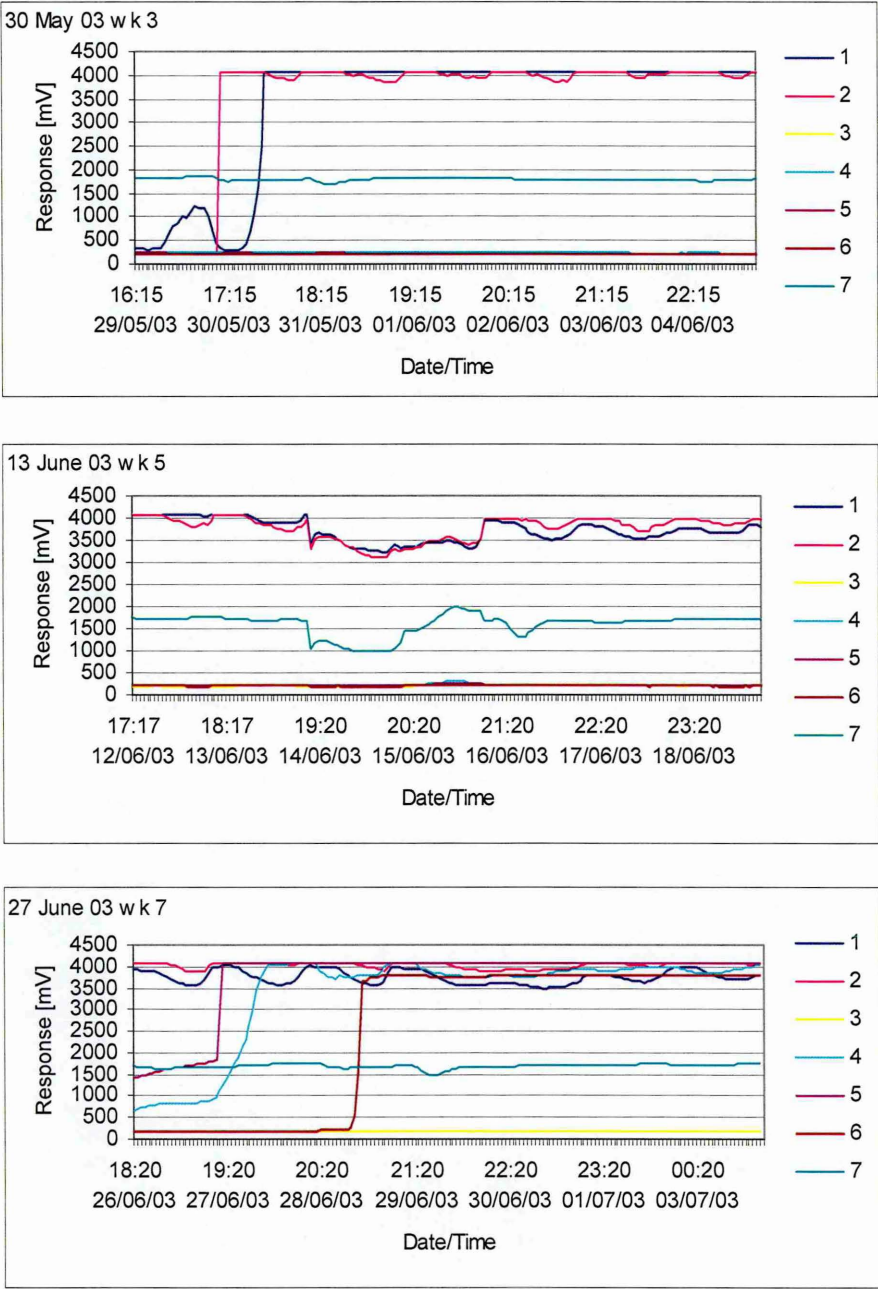


Fig. 4.38 Sensor data from lysimeter Spring/Summer trial #2 (diesel in sand)
Sensors were placed around the UST and at varying depths (3.3.3). The trial was run for 3 months. A total of 2 l diesel was leaked periodically in 0.5 l aliquots (Table 3.9). Data sampling was hourly. Results from sensor trigger events in weeks 3, 5 and 7 are shown, as indicated.

4.3.2.3 Lysimeter Spring trial #3 (petrol in topsoil)

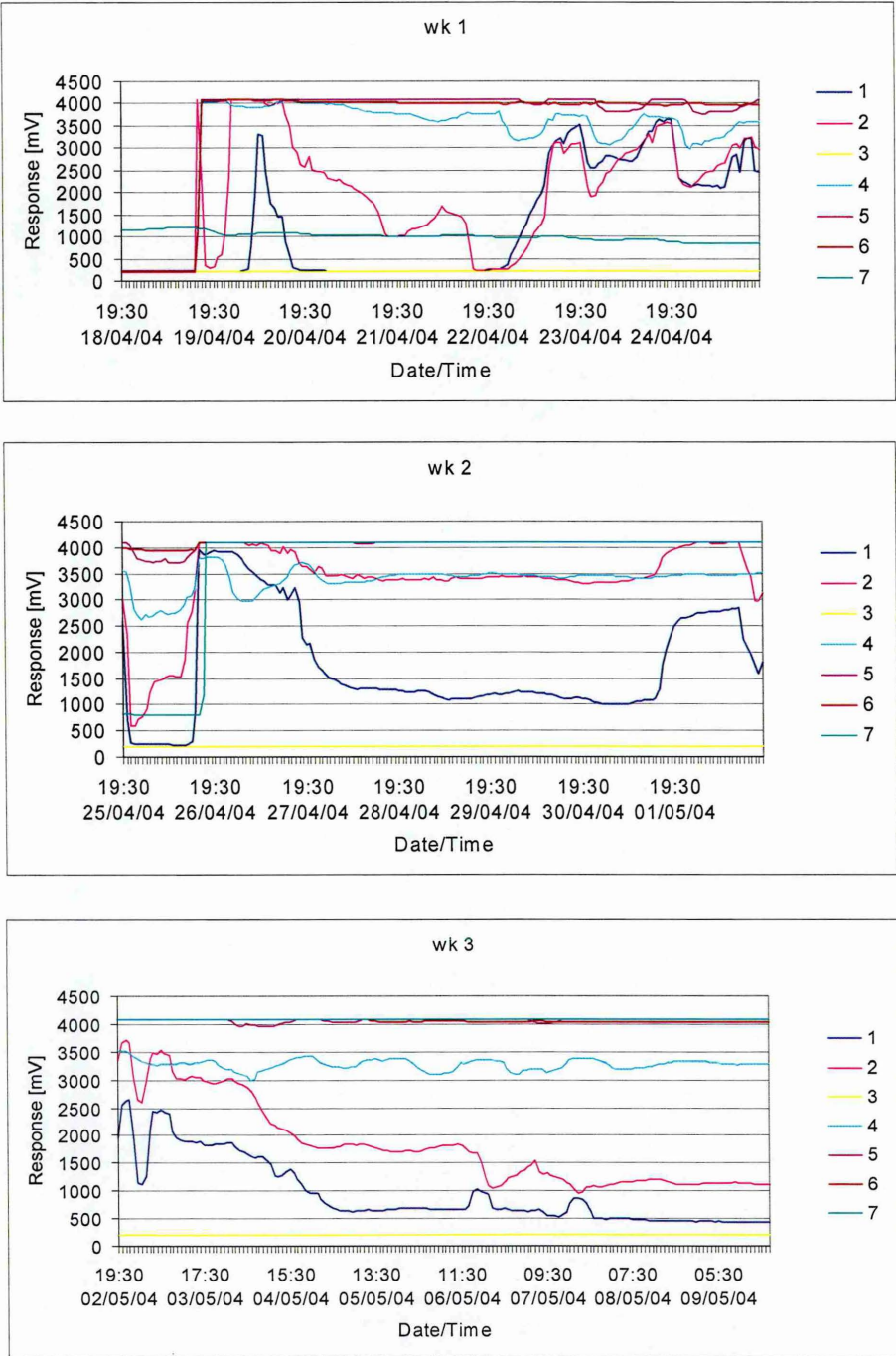


Fig. 4.39 Sensor data from lysimeter Spring trial #3 (petrol in topsoil)
Sensors were placed around the UST and at varying depths in topsoil (3.3.3). The trial was run for 4 weeks. A single 0.5 l petrol leak was initiated on 19/4/04 (Table 3.9). Data sampling was hourly. Results are shown from sensor trigger events in weeks 1, 2 and 3, as indicated.

4.3.2.4 Lysimeter Summer trial #4 (diesel in topsoil)

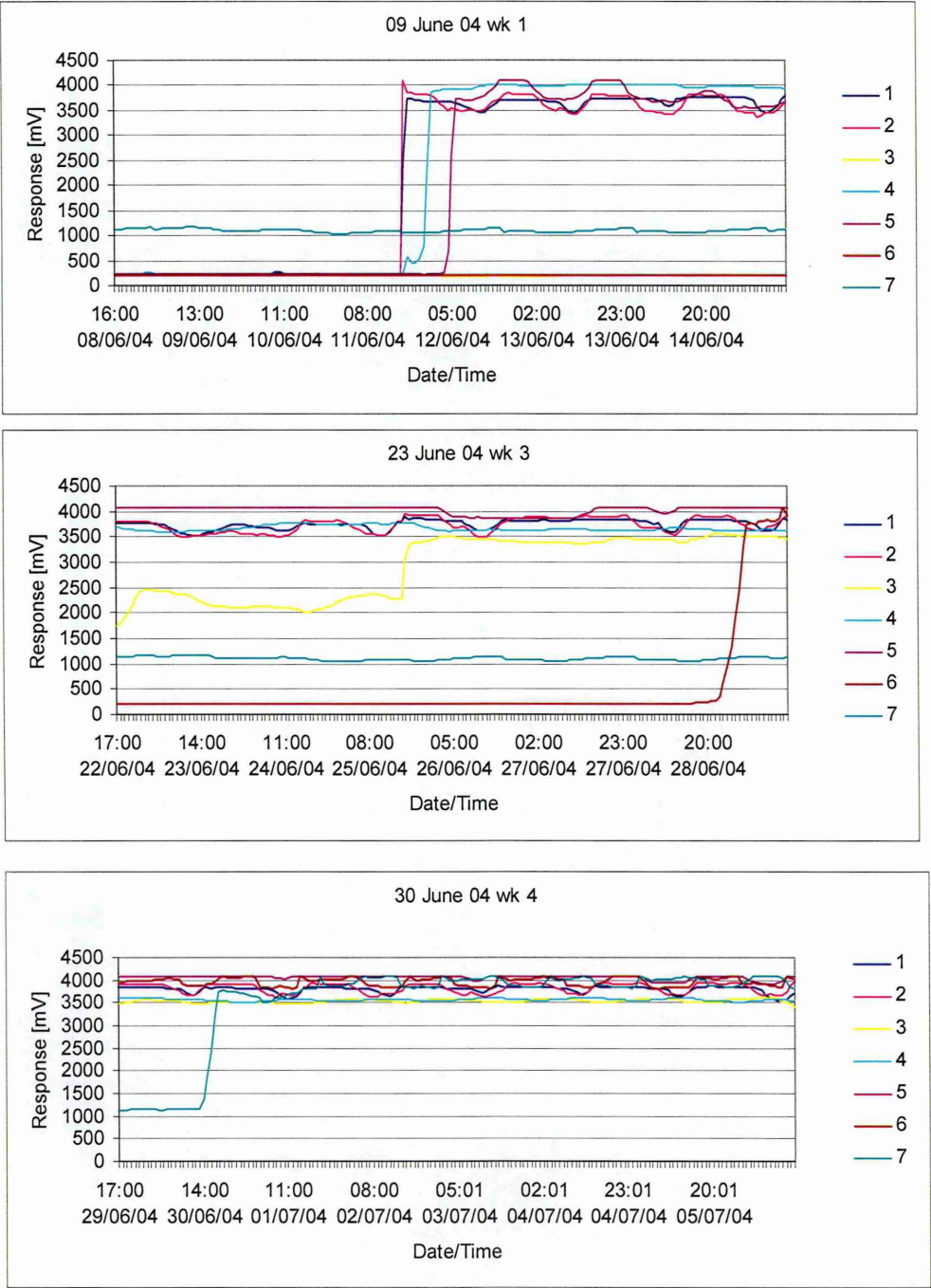


Fig. 4.40 Sensor data from Lysimeter Summer trial #4 (diesel in topsoil)
Sensors were placed around the UST at varying depths (3.3.3). The trial was run for 5 weeks. On 11/6/04, 0.5 l diesel was leaked, followed by a 2nd leak on 25/6/04 (Table 3.9). Data sampling was hourly. Results are shown from sensor trigger events in weeks 1, 3 and 4, as indicated. Signals were stable during weeks 2 and 5.

4.4 CONCLUSIONS AND DISCUSSION

Detection of fuels in water or fuels on water surfaces

In response to the environmental threat to groundwater systems posed by leaking hydrocarbon fuels (Chapter 1), the Mk 1 sub-surface sensors were evaluated as potential groundwater borehole monitoring devices. Experiments were devised to determine sensor responses to both sheared fuel/water mixtures and free-phase fuel contamination on water surfaces (4.2.1- 4.2.2). Results indicated that the Mk 1 sensors could respond to diesel, petrol and MTBE, but that sensitivity was very low, requiring 20% (v/v) fuel/water mixtures to trigger a response. Water surface tension, or cohesion between water molecules, was believed to form a barrier across the hydrophobic membrane, creating an impermeable barrier between the membrane and the fuels. Even at this high concentration, detection was only possible within turbulent water. This was attributed to the destruction of the 'surface tension barrier' allowing small, hydrophobic fuel particles access to the membrane. These fuel particles still only made random contact with the membrane surface. Similarly, the Mk 1 sensors could not detect surface water contamination (up to 200 μl fuel/44.2 cm^2 membrane surface area) regardless of how the sensor heads were positioned on the water surface. The surface film was clearly visible, yet despite having reached the sensor heads after 2-3 minutes, no fuel to membrane contact was achieved. The water surface was literally repelled from the sensor head and this surface film of water prevented any contact with the hydrophobic fuels. The only sensor response observed was when surface fuel was splashed onto air-exposed membrane, held above the water surface, which subsequently turned translucent. Hydrophobic hydrocarbons, particularly the non-aqueous phase liquids (NAPLs) can be literally trapped between water particles (Fig. 4.41).

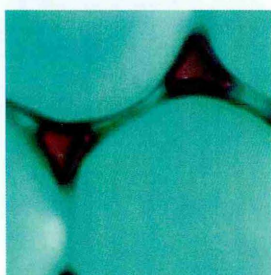


Fig. 4.41 Water droplets and NAPL particles

The figure shows water droplets surrounding small, non-aqueous phase liquid droplets (NAPLs), such as hydrocarbon fuels.

Therefore, in their current configuration, it has to be concluded that the sensors are not suitable for fuel detection in or on water. However, it is possible that the sensors could be redesigned to incorporate a miniaturised pumping device, which could draw water through the membrane. This would negate water surface tension effects by producing turbulent flow. In this way, the

organic fuels could be accumulated on the membrane surface. However, the degree of concomitant 'wash-out' by the constant throughput of water would have to be ascertained and may well prove to be counter-productive.

Evaluation of membranes

The novel aspect of the reflectometric sensors is the incorporation of the hydrocarbon-specific membrane, which turns translucent on contact with fuels. This phenomenon is a result of the natural properties of the membrane and can also be observed with paper (cellulose). Whilst paper looks opaque, it consists of microscopic transparent fibres with air trapped in-between. When light is directed at these fibres, it is refracted at each fibre-air interface, thus giving the appearance of opacity. When oil is absorbed, the air is displaced and the pores filled with oil. Oils have a refractive index closer to that of the fibres, therefore, light refraction is reduced and light can pass through, resulting in transparency (Cobb, 1994).

Membranes with different absorption and filtration properties were evaluated for potential use with the sub-surface sensors. Mk 3 sensors were fitted with each membrane and tested with a range of fuels (4.2.3). With all fuels tested, the Fluorotrans membrane (0.2 μm pore size, polyvinylidene difluoride) proved to be the most suitable membrane due to its consistent high sensitivity, greatest dynamic signal range (between contaminated and uncontaminated states) and repeatable regeneration. This spontaneous regeneration of the membrane is a very useful characteristic for field monitoring purposes as it removes the need for the sensors to be recovered (and subsequently cleaned and replaced) after a contamination incident. A typical example would be where 'pulsed' hydrocarbon (or MTBE) contamination occurs. This is when monitoring wells show irregular contamination. This is most commonly found in areas close to petrol stations, particularly with MTBE and it is generally believed that the petrol station is the source, most likely when the tanks are being refilled. The sensors (situated in or close to the petrol station) would trigger on initial contact and then could regenerate *in situ*. Even if the sensor had not completely regenerated back to baseline levels, the next contamination incident would cause another signal increase. The sensor information, with recorded dates and times of incidences, could prove very useful in tracing pulsed contamination incidents back to the source, such as the petrol station.

It was also possible to distinguish between different fuel types by monitoring the signal kinetics from the regenerating contaminated membrane. Petrol and diesel could migrate from the Fluorotrans membrane in both air and water matrices, with retention times of diesel being much higher than that of petrol. This was attributed to the more volatile and soluble components present in petrol (oxygenates). Thus, the rate of migration from the membrane distinguished

between the two fuels. This migration/membrane regeneration was also apparent in subsequent field monitoring trials (4.3.2.3). The Fluorotrans membrane was exposed to natural soil and groundwater biota, as well as to laboratory-grown organisms (4.2.6) and proved to be highly resistant to biofouling. It was also the most flexible and durable membrane, which demonstrated marked resistance to all fuels and solvents tested (MeOH, EtOH). No biofouling or deterioration of the membrane was seen after longer-term exposure to soil and fuel contaminants in the field trials.

Removal and sonication of contaminated (translucent) membranes (in 1% v/v methanol/water) liberated absorbed fuel that could then be subsequently characterised by a commercial BTEX ELISA (4.2.7). These results offer the interesting possibility of using the sub-surface sensors as an analyte collection tool, in addition to their use as a fuel leak monitor.

Effects of different soil types and conditions on sensor performance

Experiments were performed in which sensors (fitted with Fluorotrans membrane) were placed into different soils (with variable water content), which were contaminated with different fuels (4.2.5). For all soil types investigated, fuel took longer to absorb into the membrane under increasingly wet conditions, again most likely because of water molecules hindering hydrocarbon passage to the membrane. Additionally, due to the disposition of fuels to float on water (as a result of density), high water content can prevent vertical movement of fuel down through soil horizons and consequent transportation to the sensors. Fuels could not penetrate saturated clay soil and mixing merely resulted in the production of a fuel-coated clay mass. Clay is renowned for its impermeable properties due to very small fine particles (<0.002 mm opposed to 0.5-2 mm for sand) and correspondingly small pore spaces. Clay therefore forms an effectively impermeable barrier against fuels. Indeed, for this reason, clay is commonly used as a liner material in landfill sites to contain leachate. Therefore, sensor responses obtained (in saturated clay soil) were probably due to free-phase fuel on the clay surface. However, total water saturation represents a worst-case scenario for detecting fuel leaks from USTs. This degree of saturation would normally only occur in river or marine sediments. Dry or damp soils (with intermittent water input) are more common globally. All fuels could be detected at low concentration (5.6 % w/w) in damp, mixed sand/clay soil.

UST laboratory simulations

These experiments were designed to evaluate the sensors' ability to detect fuel leaks from a partially buried UST under simulated environmental conditions, and also investigated the effects of soil water flow and degree of soil saturation (4.2.4). Sand is commonly used in soil hydrology studies due to its loose structure, in which water can flow easily around the

individual soil particles. In heavier soils, particles tend to aggregate into compact structures of uneven shape, size, density and coherence and water flows preferentially along fine fissures, capillaries or pore channels. The denser the aggregates, the more likely it is that water will bypass parts of the soil matrix completely (Brown *et al.*, 1990). In both dry and damp sand, the fuel plume was shown to move vertically through the sand horizon until the water table was reached, before then being transported horizontally away from the point source. This is characteristic plume behaviour, in which contaminant plumes migrate down to the groundwater surface. Once the plume reaches the groundwater surface, it will migrate in the groundwater flow direction whilst spreading out laterally and longitudinally (Mace *et al.*, 1997, US EPA, 2005).

In fully saturated soils, soil water pressure, and subsequently hydraulic conductivity, is effectively zero. In the laboratory simulations, excessive soil water actually impeded the rate of fuel leakage from the UST and adversely affected natural fuel plume development. Results from the different sensor arrangements (using Mk 2 and 3 sensors) around the UST (and/or in the immediate vicinity) were able to provide some indication of plume migration under these conditions.

Using layers of sensors placed at increasing depth (in a vertical grid formation), it was shown that the fuels were retained within dry and damp sand and did not pass through the basal boundary into the underlying gravel/air layer. Rather, the fuel plumes (both for diesel and petrol) moved vertically down through the sand to the basal layer. This retention within the sand can be attributed to the natural suction within the sand pore spaces, caused primarily through (water) capillary action. In dry and damp sand, the pore spaces are filled with air and so water is held in finer pores and more tightly to particle surfaces, which in turn increases the soil pore water pressure (Brown *et al.*, 1990). The fuel plumes then moved horizontally through the lowest layer of sand (away from the point source) towards the water extraction point. This movement was caused through soil convection, driven by the extraction pump, which in turn generated horizontal water movement, mimicking lower-level groundwater flow.

These findings show that the amount of water present in the soil, the soil type and the water movement directly affects the potential for any sensor to detect a fuel leak. Consequently, the precise positioning of the sensors around any UST is of paramount importance and was therefore investigated further in simulated field trials (lysimeter experiments).

Field trial evaluations of the Mk 3 sub-surface sensors (lysimeter trials)

Soil lysimeters are commonly used for the study of soil hydrology and leaching studies (Brown *et al.*, 1995). Extensive lysimeter field trials using both sand and local topsoil (~50% clay) showed that the Mk 3 sensors responded rapidly to fuel leakage in both sand and soil matrices (4.3.2). In all cases, the fuel plume showed a propensity to migrate downwards through the soil horizon. Whilst there was no evidence of fuel accumulation directly underneath the UST base (indicated by a centrally placed sensor), the vertical plume migrated through the centre of lysimeter. This is common flow behaviour in lysimeter field studies and, whilst this gives a relatively accurate simulation of flow, the results are only applicable when vertical flow predominates and flows down to the groundwater table or aquifer (Brown *et al.*, 1995). The plume flow was successfully tracked by the additional sensors, placed centrally at 10 cm depth intervals. Plume flows were faster in topsoil than sand, which can be attributed to the water retention properties of each matrix. For example, with a water content of 10 cm³/100 cm³ matrix, sand has a matric suction of 10 kPa compared to ~4000 kPa for clay (Brown *et al.*, 1990). Low water retention by sand was also evidenced during the UST simulation experiments.

All sensors were controlled by the Medsci DLU, sampling every hour, and these parameters could easily be reprogrammed on-site using the simple, dedicated software provided. Collected monitoring data (over 24 hour periods) was successfully relayed back to the operator as daily email attachments. The DLU battery, powering the unit and each sensor had not depleted after 3 months of hourly sampling (conveyed by daily emails). Later developments (in conjunction with Medsci) also enabled on-line access to examine acquired sensor data (2.7.1).

Sensor performance, reliability and modifications

Variations in baseline values between the early Mk 1 and 2 sensors (between 1-1.4 V approximately) were confirmed to have no adverse impact on the sensor's responses. Manual positioning of the membrane and differences in torque applied to the threaded head assembly affected the degree of reflectance and created small variations in background signal. Background signals could vary ± 0.4 V as a result. The early Mk1 and 2 designs sensor head assemblies were also prone to clogging, due to the membrane being recessed within the sensor head and the inclusion of a protective mesh.

These issues were addressed with the modified Mk 3 sensors, in which the membrane was placed directly over the glass sensing window. This then provided the lowest baseline (background) signal possible (0.1 V) and subsequently increased the dynamic range of the sensors. Discarding the protective mesh and spacer O-ring and re-machining the sensor head

effectively brought the membrane closer to the soil surface, minimising the recess area within the head assembly and so removed the small area prone to clogging (2.5.2).

After repeated use of the Mk 3 sensors in the lysimeter-based field trials (4.3.2), some edge deterioration of the glass sensing window had occurred through repeated removal and tightening of the sensor head assemblies. Alternative plastics were evaluated as potential replacement materials, which indicated that PETg may be suitable. However, this design fault was rectified with the Mk 4 sensor design. The membrane recess area within the sensor head incorporated a brass 'lip', which then covered and protected the edge of the glass, preventing edge damage from over-tightening onto the sensor body (2.5.4). Glass was therefore retained as the material of choice for the sensing window.

Similarly, the sealant (epoxy resin) around the cable exit on the Mk 2-3 sensors deteriorated after repeated exposure to fuels. This could have been exacerbated from the physical stresses imposed through post-trial removal of the sensors from the then compacted soil within the lysimeter. This too was addressed with Mk 4 sensor design, which incorporated a strengthened cable exit assembly, which itself sealed the sensor housing, eliminating the need for internal sealant (2.5.4).

Potential future applications for the sub-surface monitoring system

Whilst the sub-surface sensors proved unsuitable for detecting the presence of hydrocarbon fuels present either in suspended or sheared in water, or as free-phase contamination on water surfaces, they can be extremely effective for detecting a range of fuels leaking through soil matrices. The reason for the difference in performance underlines the fundamental way in which these sensors work by way of absorption of fuel by a membrane.

When an array of sub-surface sensors are employed in conjunction with the Medsci DLU, the system has a proven applicability as a remote, early warning device for identification of fuel leakage in soils, for instance from underground storage tanks. However, using this particular system, this work has also shown that the sensors need to be carefully placed around a UST. Sensors should be placed close to the base and ideally where potential leak points can be identified, such as tank joints or pipe connections. The number of sensors required for effective monitoring will greatly depend on the size of the storage tank.

Another complication is that usually soils are not homogenous and consist of mixed heterogenic soils and different soil horizons, which vary in gravel/stone/clay contents, each with very different water retention properties. Obviously, the latter may not be important if sited in arid,

desert regions. Additionally, tanks may be placed above other underground utilities, for example, water and gas lines, sewers or even rubble from demolished structures. These factors will all contribute to influencing the flow of water/fuel plumes downward through the soils and will create preferential flow pathways. This can be described as a pathway capable of conveying water at a rate greater than the average linear flow velocity. Whilst the lysimeter studies were very successful in tracking the vertical flow, only 30-40% of UK soils demonstrate a vertical flow directly to the groundwater table or aquifer. Most soils predominantly have slowly permeable or impermeable layers after ~1.2 m depth, which then give rise to lateral flow, often under temporarily water-logged conditions (Hollis & Carter, 1990). Soil variability will consequently result in variability in the distribution of water and contaminants, and the ease in which they are transported. Site-specific field reports on soil classification, permeability, moisture and horizons would therefore be necessary for planning any monitoring system, and may require core samples, as well as groundwater flow and recharge rate modelling (FRTR, 2002).

In existing UST sites, optimum placement of sub-surface sensors would necessitate underground, horizontal drilling. Whilst this retrofitting would be extremely expensive, it is a nevertheless a possibility. Most USTs are placed upon a stabilising gravel bed, where fuel can easily flow into the large pore spaces between the gravel. Inserting sensors to this region through an underground pipe into this region may induce a preferential pathway by displacing the gravel/soil, and actually direct any fuel to the emplaced sensors.

A practical solution?

However, a more practical application would be to employ the sub-surface sensor system in new UST sites. The initial concept was to place an array of sensors within the gravel bed underneath the UST, which again may have aided leak detection through creating preferential pathways within the gravel/soil. However, legislation with regards to all USTs has been extensively revised. Under new UK storage guidelines (which closely follows US legislation), from 01 September 2005, all tanks storing >200 litres oil/fuel must be protected by a secondary containment system, such as a bund, capable of retaining 110% of the maximum contents (EA, 2001). However, these are generally constructed from brick or concrete (for example, petrol forecourt storage tanks) and therefore are not an absolute leak prevention method as they too can crack and leak. Above ground tanks can contain between 200-45,000 litres (Ledbury Welding & Engineering, 2004). Any underground pipework should also have leakage detection (unspecified). These systems are legally required (under the storage guidelines) to also have a leak detection system. Therefore, there is still a clear requirement for the sub-surface sensors, which can be employed in a number of different scenarios.

One solution might be found in the emplacement of a sub-UST (possibly sub-soil surface) bund, which would be designed to guide any leakage directly to the sensors. Indeed, bund and sensors array could be designed and installed as an integrated composite system. The bund base could be designed with small, 1cm deep channels leading to a central recess, where a single sensor could be placed. Any fuel leak from the tank would flow to the central recess and contact the integrated sensor. As previously demonstrated, the sensors can respond to 8 μ l fuel on the membrane surface and so a leak would be very rapidly detected before large fuel losses were incurred. This interesting possibility has indeed been discussed with an established storage tank company, Fosse Liquitrol Ltd (Leicester, UK) who believe that it is both technically and economically feasible to manufacture such an integrated system. This technique can be also employed for above-ground storage bunds, which is now the official EA-approved storage method.

Many UST tanks are monitored for leakage using internal fuel level gauges. However, such gauges are insensitive, for example, a leak rate of 1 US barrel per hour in a 100 ft diameter tank will produce a level change of ~ 0.1 inch per hour (Miller & Brown, 1988). An alternative could also be to place sensors with the interstitial spaces of double-walled storage tanks. Any leakage would accumulate in the base of the interstitial space. This could be extended to include oil tanker ship hulls, which are now required to have an outer hull, spaced from the inner hull carrying the fuel/oil, which acts as a second skin, for safety purposes. Most large pipelines are also double-walled. A 'Soil sentry liquid 330 system' (Phoenix Technologies Corp., Arizona, USA) is marketed as an interstitial dry/wet optical probe for monitoring leaks in such systems, based on IR refraction. However, this device cannot differentiate between water or hydrocarbons. The sub-surface sensor (incorporating the hydrophobic Fluorotrans membrane) would be specific to hydrocarbons and has the ability to regenerate.

Whilst not particularly novel, another alternative application for the sensors could also be as cheap and reliable detection devices for sensing fuel overfills in storage tanks or road transporters. Fig. 4.42 shows two marketed overfill sensors used for this purpose.

This concludes the development work for the sub-surface monitoring system. The following work relates to the development of a novel immunoassay for the detection of fuel oxygenates, initially for MTBE, which is described in Chapters 5-6, which can be used in conjunction with the sub-surface leak detection system as an integrated monitoring system (Section 1.4.2).

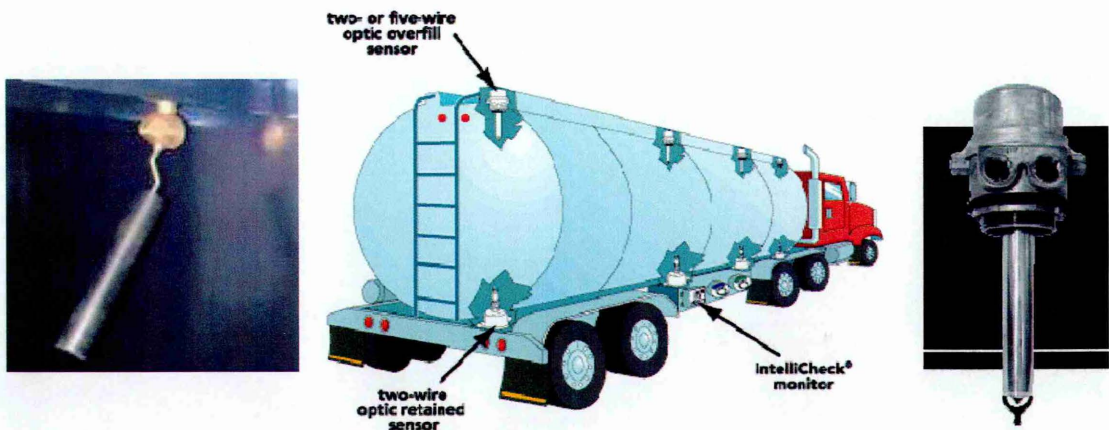


Fig. 4.42 Commercial over fill protection devices

A storage tank overfill protection device is shown on the left (Berry, Ledbury Welding and Engineering, 2004). An oil tanker 5-wire optical overfill sensing system showing internal sensors far right) and external IntelliCheck® electronic monitoring system. The device uses pulsed light signals and the refractive indices are calculated by the monitoring circuitry (Skully Signal Co., Wilmington, USA).

CHAPTER 5

MATERIALS AND METHODS

(MTBE Immunoassay Development)

5.1 MATERIALS

5.1.1 ELISA

5.1.1.1 General Reagents

Unless otherwise stated, general reagents were obtained from Sigma-Aldrich Ltd., Poole, Dorset, UK.

Buffers: Blocking buffer (BB) was prepared using Bovine Serum Albumin (BSA) in RO water at either 1 or 5% (w/v) concentrations. Phosphate buffered saline (PBS - 10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4) was prepared by dissolving 1 tablet in 200 ml RO water (Sigma P-4417 product specifications). Sodium phosphate buffer (PB - 10 mM, pH 7.4) was prepared by dissolving 1.15 g/l anhydrous Na_2HPO_4 with 0.23 g/l anhydrous NaH_2PO_4 . The pH was adjusted if necessary with 1 M hydrochloric acid (HCl) or 1 M caustic soda (NaOH). Tris (hydroxymethyl) methylamine (Tris - 10 mM, pH 7.4) was prepared by dissolving 1.21 g/l in RO water and adjusted with H_2SO_4 . Buffer standards: pH 4 (phthalate), pH 7 (phosphate) and pH 9.2 (borate) were obtained from Fisher Scientific UK Ltd., (Loughborough, UK). RO water was prepared in-house by ion exchange and reverse osmosis (Elgastat Group, High Wycombe, Bucks, UK).

Chromogen: TMB (3,3',5,5'-Tetramethylbenzidine) for ELISA (TMB solution) was supplied as a ready-to-use solution of chromogen, hydrogen peroxide and buffer. TMB Liquid Substrate System (containing 1.58 M TMB) was supplied by Sigma-Aldrich (Poole, UK). Stable Liquid TMB (2.08 M) was supplied by Moss Inc., USA (TMBUS). Ultrasensitive TMB (2.08 M) was supplied by Europa Bioproducts Ltd., (Ely, UK). The TMB reactions were stopped with 0.1 M sulphuric acid (H_2SO_4), unless stated otherwise. Free HRP (Type 1 peroxidase, 5,000 units) was obtained from Sigma-Aldrich.

ELISA plate washing: Washing buffer (WB) was RO water containing 0.1% (v/v) polyoxyethylenesorbiton monolaurate ('Tween 20', Sigma-Aldrich).

5.1.1.2 Immunochemical reagents

Antigen (Ag): BSA-MTBE Ag conjugate (1 mg/ml) and BSA-MODMO Ag conjugate (0.607 mg/ml) were obtained from Diaclone SAS, Besançon, France.

Primary antibodies (PAb): Purified primary IgG monoclonal (mouse) antibody (1 mg/ml) and biotinylated primary IgG monoclonal (mouse) antibody (1 mg/ml) raised against BSA-MODMO were obtained from Diaclone SAS, Besançon, France.

Secondary antibodies (2Ab-HRP): Polyclonal IgG goat anti-IgG mouse antibody-HRP conjugates (2 mg/ml) were obtained from AB-CAM Ltd., Cambridge, UK.

Antigens and antibodies were aliquoted (100 µg quantities) on receipt and stored at -20 °C. Aliquots were thawed at RT (22 °C) immediately prior to use. Any unused fractions were then stored at 4 °C and used within 1 week. Dilutions were made using PBS (10 mM, pH 7.4) as described in 5.2.1.2.

Peroxidase-conjugated streptavidin): Streptavidin-HRP conjugate (s-HRP, 0.63 mg/ml) was obtained from Dakocytomation Ltd., (Ely, UK). A second s-HRP conjugate ('Prozyme', 1 mg/ml) was obtained from Europa Bioproducts Ltd., (Ely, UK). These were both stored at 4 °C (manufacturers' specification).

5.1.1.3 Fuels and organic chemicals

Unleaded petrol and diesel was obtained as described in 3.1. All other chemicals were spectroscopic-grade (99.9%). The 7' Methoxy-3,7-dimethyloctanal (MODMO) was obtained from Acros organics (Fisher Scientific UK Ltd., Loughborough, UK). Straight-chain alcohols - 1-butanol, 1-propanol, 1-pentanol, 1-hexanol, 1-octanol, 2-propanol, 2-methyl, 1-butanol, 2-methyl, 1-propanol, 2-methyl, 1-pentanol, 2-methyl, 2-butanol; 2-methyl, 2-pentanol; 3-methyl, 1-butanol and 3-methyl, 1-pentanol; were obtained from Sigma-Aldrich (Poole, Dorset, UK). Fuel oxygenates; *tert* amyl methyl ether (TAME), ethyl *tert* butyl ether (ETBE), *di*-isopropyl ether (DIPE), *tert* butyl ether (TBA) & *tert* butyl formate (TBF), along with two oxygenate alternatives; ethanol (EtOH) and methanol (MeOH) were also obtained from Sigma-Aldrich.

Unless otherwise stated, mixtures of MTBE and these other organic compounds (0-5000 ppm) were made with PBS (10 mM, pH 7.4) in 25 ml polycarbonate tubes (5.1.1.5) prior to analysis.

5.1.1.4 Groundwater samples

Groundwater samples were obtained from the following European sites:

- Stadtwerk AG Wasserwerks, Düsseldorf, Germany
- Technische Universität Dresden, Pirna, Germany
- SENSPOL Technical Meeting (2003), Bundesanstalt für Gewässerkunde (BFG) and Tübingen University, Koblenz, Germany
- Salzburg AG Centre Wasser, Salzburg, Austria
- Cranfield University, Silsoe, UK (on-site groundwater monitoring well)

5.1.1.5 Apparatus

‘Maxisorp’ F8 96-well flat-bottomed microtitre plates were used for all ELISAs (Merck Eurolab, Poole, UK). A manual 8-channel washer (A/S Nunc, Roskilde, Denmark) was used for plate washing steps. Each well was filled with ~300 µl WB per wash.

All dilutions of Ag, antibody and organic mixtures were freshly made prior to analysis in 25 ml polycarbonate tubes (Fisher Scientific UK Ltd., Loughborough, UK).

Absorbance (4450) was read using an automated microtitre plate reader (Thermo Labsystems, Helsinki, Finland) using MRX Revelation 2.0 software.

5.1.2 Protein conjugations

5.1.2.1 General reagents

Unless otherwise stated, general reagents were obtained from Sigma-Aldrich Ltd., Poole, Dorset, UK.

Buffers: Blocking buffer (BB) was prepared by dissolving Bovine Serum Albumin (BSA) in RO water at either 1 or 5% (w/v) concentrations. PBS and sodium phosphate buffer (10 mM, pH 7.4 or 6.3 respectively) were prepared as 5.1.1.1. Borate buffer (0.2 M, pH 7.7 & 9.2) was prepared by dissolving 4.02 g sodium tetraborate in 100 ml RO water and adjusted with 1 M HCl. (5.1.1.1).

5.1.2.2 Conjugation reagents & apparatus

The native primary antibody (PAb) and antigen (BSA-MODMO) used in these experiments are the same as in 5.1.1.2. Maxibind™ BSA (10 mg) was obtained from Uptima/Interchim, France. Free HRP (Type 1 peroxidase, 5,000 units) was obtained from Sigma-Aldrich, Poole, UK. A

proprietary BSA-MODMO-HRP conjugate was obtained from Diaclone SAS, Besançon, France. MODMO was obtained as stated in 5.1.1.3. Dimethyl formamide (DMF) was obtained from Sigma-Aldrich, Poole, Dorset, UK.

Sodium *meta*-periodate (NaIO_4) and sodium hydroxide (NaOH) were obtained from Fisher Scientific UK Ltd., (Loughborough, UK). Glycoprotein Detection Reagent was obtained from Pierce (Perbio Science UK Ltd., Tattenhall, UK). Sodium cyanoborohydride (NaCNBH_3) and anhydrous sodium tetraborate (coupling buffer) were obtained from Acros Organics (Fisher Scientific UK Ltd., Loughborough, UK).

Dialysis: Dialysis of all conjugates was made using Pierce 'Slide-A-Lyzer' dialysis cassettes (0.5-3 ml capacity) with a 10,000 kDa. molecular weight cut-off, with Pierce buoys and 1 ml syringes with 18-gauge needles (Perbio Science UK Ltd., Tattenhall, UK).

Conjugations: Conjugation mixing was performed in 2 ml tubes placed onto a Dynal 'MX1 12-tube Sample Mixer' set at 18 rpm (Dynal Biotech, Wirral, UK). Microtitre plates were shaken (where appropriate) using a standard plate shaker.

5.1.2.3 Conjugate assays

Microtitre plates: Nunc 'Maxisorp' F8 96-well flat-bottomed microtitre plates were used as described in 5.1.1.4. Pierce 'Reacti-bind' antibody-coated plates (goat anti-IgG mouse) were obtained from Perbio Science UK Ltd., Tattenhall, UK.

Washing steps: Washing steps were performed using washing buffer (RO water containing 0.1% (v/v) Tween 20) as for the ELISAs described in Protocol 3 (5.2.1.6) using a manual 8-channel washer (5.1.1.5).

Colour substrate: The chromogen 'Ultrasensitive TMB' (2.08 M, Europa Bioproducts, Ely, UK) was used throughout these experiments.

5.1.3 Enzyme-linked flow-through assays (ELIFA)

Reagents: The antibodies and Ag used in these experiments are the same as in 5.1.1.2. BSA-MODMO Ag and biotinylated PAb were prepared as in the ELISA Protocol 2 (5.2.1.4); native PAb, and 2Ab-HRP were prepared as in ELISA Protocol 3 (5.2.1.6) unless otherwise stated.

Blocking buffers (BB): Superblock™ Blocking Buffer in PBS (pH 7.4) was a proprietary protein formulation and was used undiluted according to the manufacturer's instructions (Pierce - Perbio Science UK Ltd., Tattenhall, UK). Blocker Casein (1% w/v) in TBS was also supplied by Pierce. A stock solution of BSA blocker (2% w/v) was prepared in RO water. BSA, PBS tablets, Tween 20 and TMB Liquid Substrate Solution (TMB) were supplied by Sigma (5.1.1.1). PBS (10 mM, pH 7.4) was prepared by dissolving one tablet in 200 ml RO water. Any buffer dilutions were made in RO water.

A single MTBE solution (5000 ppm) was prepared in PBS (5.1.1.3), MTBE was obtained from Acros Organics (Fisher Scientific UK Ltd., Loughborough, UK).

Membranes: Hybond+ Nylon, Biodyne+, and Immunodyne were supplied by Pall-Gelman (UK). DEAE-cellulose (DE81+) was obtained from Whatman International Ltd., (Maidstone, UK). Charged membranes are indicated by a + (positive charge) or – (negative charge) symbol. Immunodyne was a neutral membrane, which has a proprietary latent chemistry, which forms covalent bonds with amines. It is activated by wetting, and so was stored under nitrogen. Membranes were all 0.45 µm pore size. Membranes were cut into 6 mm dia. discs prior to use, handled only with fine membrane forceps and stored in plastic Petri dishes.

Equipment: Standard plastic Petri dishes were used for Petri dish incubations. Plates were shaken on a 'Lab-Line Titre Plate Shaker' (Fisher Scientific UK Ltd., Loughborough, UK) on the lowest setting (100 rpm). Custom perspex flow cells were designed by Dr John Bolbot (Cranfield, UK) and manufactured by Model Products Ltd (Wooton, UK). Through-flow was generated using a Minipuls™ 4-channel peristaltic pump (Gilson S.A.S, France). PVC peristaltic pump tubing (1.0 mm) was used throughout (Anachem, Luton, UK).

5.1.4 Magnetic bead immunoassays

5.1.4.1 Reagents

Dynabeads® M-280 Tosylactivated and Dynabeads® M-270 Epoxy were obtained from Dynal Biotech (Wirral, UK).

Ag (BSA-MODMO), PAb and 2Ab-HRP reagents were the same as described in 5.1.1.2. The colour substrate TMB was supplied by Moss (5.1.1.1). MTBE and MODMO were as described previously (5.1.1.3).

Buffers: The following buffers were prepared and used throughout this development: Buffer A - Sodium phosphate (0.1 M, pH 7.4), Buffer B – Borate (0.1 M, pH 9.5 was adjusted with 5 M NaOH), Buffer C – PBS and 0.1% w/v BSA (0.01 M, pH 7.4), Buffer D – Tris (0.2 M, pH 8.5) and 0.1% w/v BSA. Buffer C was also used throughout as a wash buffer (WB), unless stated otherwise. Tween 20 (T20) was as described in 5.1.2.3.

5.1.4.2 Equipment

A Dynal 'MPC' 6-tube magnetic rack and Dynal Sample Mixer (10 rpm slow tilt rotator) was obtained from Dynal Biotech (Wirral, UK). A standard vortex machine was used for resuspending supplied Dynabeads.

Larger solution volumes were made in 25 ml polycarbonate tubes (Fisher Scientific UK Ltd., Loughborough, UK). Smaller volumes were made in 2.5 ml Eppendorf tubes.

The absorbance of coloured product (TMB) was determined using the plate reader and microtitre plates described in 5.1.1.5.

5.2 METHODS

This Section is divided into 4 main parts: each details the protocols and experimental work during development of the ELISA (5.2.1), protein conjugations, (5.2.2) the transposition of the ELISA into (i) an enzyme-linked *flow-through* assay (ELIFA, 5.2.3) and (ii) magnetic bead immunoassay format (5.2.4).

5.2.1 ELISA

5.2.1.1 Data manipulation

5.2.1.1.1 LOD

Best-fit straight line, linear regression equation and R^2 values were calculated for the standard curve obtained using Microsoft Excel. The LOD calculated from the linear regression equation was defined as the MTBE concentration which gives a mean signal (y) equal to the blank (y_b , zero MTBE) plus three standard deviations of the blank ($y = y_b + 3SD$).

5.2.1.1.2 Immunoassay data

MTBE immunoassay data was generally reported by plotting the mean absorbance (4450) against known concentrations of analyte. Where appropriate, this was also reported as % B/Bo against analyte concentration.

The % B/B₀ was the assay signal (mean A₄₅₀) obtained for a given sample (B) reported as a percentage of the assay signal (mean A₄₅₀) obtained from a zero diluent or blank (B₀). As the assay signal was inversely proportional to the amount of analyte in the sample (darker colour with higher absorbance indicated lower analyte concentration), the % B/B₀ value decreased as the concentration of sample analyte increased.

The % B/B₀ for known BTEX concentrations (obtained using the SDI Total RaPID BTEX Assay Test Kit, Section 1.5.2.2) were plotted on a logit ordinate (y axis) against BTEX concentration on a logarithmic abscissa (x axis) to obtain a typical immunoassay calibration curve. The % B/B₀ values of “unknown” assayed samples were read against this calibration curve and the BTEX concentration thus determined. Values <0.09 ppm or >3 ppm were reported as either under or over these values. The full protocol is given in Appendix 3.

5.2.1.2 Protocol 1 for ELISA using 96-well microtitre plates

The development of the MTBE ELISA starts from Protocol 1, which was based on a generic IA protocol. This was then optimised with respects to several variables. Protocol 2 was the result of these optimisations. Protocol 3 was the result of further optimisation and was subsequently used for further investigations and field trials.

General assay practice: All reagents were brought to RT (22 °C) prior to use. Assay solutions; Blocking Buffer (BB), Ag, antibodies and analyte; were freshly prepared in 25 ml polycarbonate tubes prior to use (Table 5.1). These solutions (including the TMB colour substrate solution and stop solution - 1 M H₂SO₄) were poured into clean Petri dishes (as and when required during the assay procedure) and immediately dispensed into plate wells using an 8-channel pipette. Any surplus was then discarded.

MTBE (analyte) solutions: Serial dilutions of MTBE (0.0005-5000 ppm) were prepared from a fresh stock solution (Table 5.1) with PBS (5.1.1.3), 30 min. prior to use.

Washing steps: Washing steps with wash buffer (WB, RO water with 0.01% v/v Tween 20) were performed with an 8-channel manual washer (300 µl/well). Plates were inverted onto absorbent paper and tapped to remove any residual between each wash step.

Table 5.1 Reagent dilutions (Protocol 1)

Reagent stock solutions and dilutions are shown in order of use within the ELISA procedure. Washing steps have not been indicated.

Reagent	Stock concentration	Dilution Factor	Diluent	Volume/well (μl)	Final concentration
BSA-MTBE Ag	1 mg/ml	1:2000	PBS ²	100	50 ng/well
BSA-MODMO Ag	0.106 mg/ml	1:212	PBS	100	50 ng/well
BB (BSA)	BSA concentrate	5:100	RO water pH 7.7	250	5% (w/v)
Biotinylated PAb monoclonal	1 mg/ml	1:1000	PBS	100	100 ng/well
MTBE (analyte) stock "sample" solution	0.744g/ml (99.8% pure)	1:149	PBS	100	5000 μg/ml (ppm)
S-HRP ¹	0.63 mg/ml	1:5000	PBS	100	126 ng/ml
TMB solution	1.58 M	None	None	100	1.58 M
Stop solution H ₂ SO ₄	18 M	1:18	RO water pH 7.7	100	1 M

¹ The minimum manufacturer's specification for ELISA

² PBS (10 mM, pH 7.4)

96-well plate preparation

All experiments were performed with Maxisorp 96-well plates (5.1.1.5) in stationary conditions and at RT (22 °C), unless specified otherwise.

Ag coating: The BSA-MTBE Ag and BSA-MODMO Ag were diluted as shown in Table 5.1. For the coating of plate wells (with either Ag), a 100 μl volume was dispensed into each well (Table 5.1). The plates were sealed with acetate film and incubated overnight at 4 °C.

Blocking step: Ag-coated plates were washed four times with WB. Wells were blocked with 250 μl BSA solution (5% w/v), sealed with acetate and incubated for 2 hr at RT. Plates were then washed twice with WB, air-dried for 10 min., sealed again with acetate and stored at 4 °C in an airtight container, containing Mini-Pax™ indicating silica absorbent (Sigma-Aldrich, Poole, Dorset, UK).

Assay procedure

Ag-coated plates were brought to RT for 1 hr prior to use. All reagents were prepared as shown in Table 5.1. Controls lacking Ag and/or biotinylated PAb were included in each set of experiments.

1. *Biotinylated PAb incubation (with or without analyte):* A 100 μl volume of biotinylated PAb was added to each well (100 ng/well). A 100 μl volume of analyte solution (containing MTBE), or a control solution containing no MTBE, was dispensed into plate wells

immediately *after* the addition of biotinylated PAb. Plates were sealed with acetate film and incubated for 1 hr at RT. The plate was then washed four times with WB.

2. *Secondary incubation:* A 100 μ l volume of s-HRP was added to each well (126 ng/well). Plates were sealed with acetate film and incubated for 30 min. followed by a further four wash steps.
3. *Colour development and detection:* Unless specified otherwise, 100 μ l TMB solution (1.58 M) was added to each well and incubated in darkness for 20 min. at RT. The reaction was stopped by the addition of 100 μ l Stop solution (1 M H₂SO₄). Absorbance was immediately read at 450 nm.

5.2.1.3 Step-by-step optimisation of Protocol 1 for ELISA

Protocol 1 (5.2.1.2) was optimised with respect to several variables, as indicated in each experiment below. These experiments are listed chronologically and as a result of each, the optimised parameter was maintained throughout the subsequent experiments, unless specified differently.

5.2.1.3.1 Preliminary ELISA using BSA-MTBE and BSA-MODMO Ag conjugates

Preliminary assays were performed with a single MTBE concentration (5000 ppm in PBS) as shown in Table 5.1. BSA-MTBE and BSA-MODMO Ag-coated plates were compared. Controls lacked either MTBE, or Ag, or biotinylated PAb. Non-specific binding of both primary and secondary antibodies was investigated by using wells lacking both Ag and BB. The assay was conducted as Protocol 1 (5.2.1.2).

5.2.1.3.2 Antibody-Ag binding in different substrates

As a result of 5.2.1.3.1, only BSA-MODMO Ag-coated plates were used to investigate Ab-Ag binding when the PAb (100 μ l) was mixed with 100 μ l volumes of four different substrates; PBS, RO water, 5% BSA solution and PBS containing MTBE (5000 ppm). Controls lacked Ag or PAb. The assay was otherwise conducted as described in 5.2.1.2.

5.2.1.3.3 Optimisation of BSA BB concentrations (plate preparation)

Assays were performed with BSA-MODMO Ag-coated plates blocked at either 1 or 5% BSA (w/v). Assays were then performed using 4 substrates; PBS, 5% BSA solution, RO water and PBS containing MTBE (5000 ppm). Controls lacked biotinylated PAb. In contrast to the first group of experiments (and Protocol 1), a 100 μ l volume of “sample” (PBS $\pm$ MTBE, BSA, RO

water) was placed into plate wells. Once all the wells were filled, then the biotinylated PAb (100 µl per well) was added. The assay was continued as 5.2.1.2.

5.2.1.3.4 Optimisation of assay signal: colour development

5.2.1.3.4.1 Comparison of assay signal at two absorbency wavelengths

Taking into account modifications from 5.2.1.3.1 & 5.2.1.3.3, the assay was otherwise conducted as described in Protocol 1 (5.2.1.2), to the colour development stage. After the TMB incubation step (20 min. colour development), stop solution was only added to half of the wells. The absorbance was then immediately read at 630 nm for the kinetic readings and 450 nm for the stopped wells.

5.2.1.3.4.2 Determination of optimal TMB incubation time

Assays were then conducted up to the colour development step. A 100 µl volume of TMB was added to the wells. One row (8 wells) was immediately stopped with 100 µl stop solution and the absorbance recorded (A450). Each remaining row was subsequently stopped and read after successive 5 min intervals for a total colour development time of 30 min.

5.2.1.3.4.3 Comparison of two TMB-based liquid substrate products

An alternative TMB substrate solution ‘Stable Liquid TMB’ (2.08 M, Moss Inc., USA) was compared to the ‘TMB Liquid Substrate Solution’ (1.58 M, Sigma-Aldrich, Poole, UK) used in 5.2.1.3.1-5.2.1.3.4. Maximum signals were determined in assays lacking MTBE. A single concentration of MTBE (5000 ppm) was used to determine the minimum assay signal. Controls lacked biotinylated PAb. The rates of colour development were visually compared and the final absorbance (A450) read after 10 min. Stop solution (H₂SO₄) was increased to 2 M concentration. Assays were otherwise conducted as Protocol 1, incorporating modifications from 5.2.1.3.1-5.2.1.3.3.

5.2.1.3.5 Effects of salts on biotinylated PAb-Ag binding

PBS (100%) was made by diluting 1 standard Sigma PBS buffer tablet (5.1.1.1) in 200 ml RO water (PBS contained 10 mM phosphate, 137 mM NaCl & 2.7 mM KCl). Biotinylated PAb was incubated for 1 hr in a range of PBS concentrations (0-150% PBS, pH 7.4). The assay was then conducted following Protocol 1 (5.2.1.2), incorporating modifications up to and including 5.2.1.3.4 (Table 5.2).

5.2.1.3.5.1 Effect of saline on ELISA performance

Further full-plate assays were performed with a full range of MTBE solutions (0.0005-5000 ppm in either PB or PBS). Assay PAb and s-HRP incubations were in PB (10 mM, pH 7.4) containing either 0, 50 or 100% of the usual salt content present in solutions made using standard Sigma PBS buffer tablets (5.2.1.3.5). The assays were then run as Protocol 1, incorporating modifications up to and including 5.2.1.3.4 (Table 5.2).

5.2.1.3.6 Biotinylated PAb and Ag concentrations

Checkerboard ELISAs were performed in order to determine the optimal biotinylated PAb and Ag working concentrations. A range of Ag concentrations (0 -1000 ng/ml) were coated onto 96-well plates across the wells (1-r) and blocked with 1% (w/v) BB. Biotinylated PAb was serially diluted from 1000-0 ng/ml and was different dilutions added (rows A-H) to the Ag-coated wells. The assay procedure was otherwise conducted as described in Protocol 1 (5.2.1.2), incorporating modifications from 5.2.1.3.1-5.2.1.3.5.

Further assays were performed using the full range of MTBE solutions (0.005-5000 ppm in PBS). Two Ag concentrations (250 & 500 ng/ml), blocked with 1% BB (w/v), were tested to evaluate the each assay's response to MTBE. Controls lacked PAb. Assays were performed following the modified Protocol 1 (5.2.1.3.1-5.2.1.3.5).

5.2.1.3.7 Combined incubation of biotinylated PAb and s-HRP

Ag-coated plates (50 ng/well) were prepared and coated with an increased volume (300 µl) of 1% BB (w/v). The incubation steps for the biotinylated PAb and the s-HRP were combined. The full range of MTBE solutions (0.005-5000 ppm in PBS) were added to the wells (100 µl/well), followed by the primary Ab (100 µl) and s-HRP (100 µl). The plate was incubated at RT for 1 hr, followed by 4 wash steps. TMB (100 µl) was added and left for 10 min. The reaction was then stopped and the absorbance read (A450). A second assay was performed as the modified Protocol 1 (5.2.1.3.1-5.2.1.3.6).

5.2.1.3.8 Repeatability of the ELISA using the modified Protocol 1

Full-plate assays were conducted following the optimised Protocol 1 (incorporating modifications shown in Table 5.2) to determine repeatability and accuracy of the ELISA. Ag-coated plates, prepared separately (2 week intervals) had been sealed and stored at 4 °C. These were brought to RT (22 °C) prior to use. A full range of MTBE solutions (0-5000 ppm in PBS) was used. Controls lacked PAb.

Table 5.2 Summary of the ELISA Protocol 1 optimisation

Experimental parameters, conditions applied and those retained have been summarised in the table and cross-referenced to the appropriate experimental Section.

Section	Parameter Studied	Conditions applied	Conditions retained
5.2.1.3.1	<i>BSA-MTBE & BSA-MODMO Ag conjugate effectiveness</i>	Testing of PAb binding to BSA-MTBE and BSA-MODMO Ag	BSA-MODMO Ag conjugate for MTBE ELISAs
5.2.1.3.3	<i>BSA BB concentrations (% w/v)</i>	0 – 5% BSA (w/v)	1% (w/v) BSA for blocking wells
5.2.1.3.4	<i>TMB colour development</i> Absorption wavelength TMB substrate solution comparison (M) Stop solution (H ₂ SO ₄)	630 nm (kinetic) 450 nm stopped) 1.58 M and 2.08 M Originally 1 M	A450 nm 2.08 M TMB solution (TMBUS, 100 µl/well) Stop solution (H ₂ SO ₄) increased to 2 M (100 µl/well)
5.2.1.3.5	<i>Salinity in PBS buffer (10 mM, pH 7.4)</i>	0-150 mM PBS 0, 50% and 100% saline	10 mM PBS, pH 7.4, 100% original saline content
5.2.1.3.6	<i>Ag and biotinylated PAb concentrations:</i> Ag (BSA-MODMO) Biotinylated PAb	 1-1000 ng/well 1-1000 ng/well	 50 ng/well 100 ng/well
5.2.1.3.7	Incubation of biotinylated PAb and s-HRP	Combined incubation (1 hr) of PAb and s-HRP	Separate 1 hr. incubation (PAb); 30 min incubation of s-HRP

5.2.1.4 Protocol 2 for the MTBE ELISA on 96-well plates

Protocol 2 is the result of the optimisation of the initial Protocol 1 through steps 1-8 (5.2.1.3.1-5.2.1.3.8). Optimised parameters are summarised in Table 5.3 with the detailed procedure as follows:

General assay practice: All reagents were brought to RT (22 °C) prior to use. Assay solutions (BB, Ag, antibody and analyte) were freshly prepared in 25 ml polycarbonate tubes prior to use. All solutions were poured into clean Petri dishes (as and when required during the assay procedure) and immediately dispensed into plate wells using an 8-channel pipette. Any surplus was then discarded.

MTBE (analyte) solutions: Serial dilutions of MTBE (0.0005-5000 ppm) were prepared from a fresh stock solution (Table 5.1) with PBS (5.1.1.3), 30 min prior to use.

Washing steps: Washing steps with wash buffer (WB, RO water with 0.01% v/v Tween 20) were performed with an 8-channel manual washer (300 µl/well). Plates were inverted onto absorbent paper and tapped to remove any residual between each wash step (as Protocol 1).

Table 5.3 Revised reagent dilutions (Protocol 2)

Reagent stock solutions and dilutions are shown in order of use within the ELISA procedure for the revised Protocol 2. Washing steps have not been indicated.

Reagent	Stock concentration	Dilution Factor	Diluent	Volume/well (µl)	Final concentration
BSA-MODMO Ag	0.106 mg/ml	1:212	PBS ²	100	50 ng/well
BB (BSA)	BSA concentrate	1:100	RO water pH 7.7	250	1% (w/v)
Biotinylated PAb	1 mg/ml	1:1000	PBS	100	100 ng/well
MTBE (analyte) stock solution	0.744g/ml (99.8% pure)	1:149	PBS	100	5000 µg/ml (ppm)
s-HRP ¹	0.63 mg/ml	1:5000	PBS	100	12.6 ng/well
TMB solution	2.08 M	None	None	100	2.08 M (TMBUS)
Stop solution H ₂ SO ₄	18 M	2:18	RO water pH 7.7	100	2 M

¹ The minimum manufacturer's specification for ELISA

² PBS (10 mM, pH 7.4)

96-well plate preparation

Ag coating: Experiments were performed with Maxisorp 96-well plates in stationary conditions, unless otherwise specified. *BSA-MODMO Ag conjugate* was diluted 1:212 (Table 5.3) and a 100 µl volume was dispensed into each well (Table 5.3). The plates were sealed with acetate and incubated overnight at 4 °C.

Blocking step: Ag-coated plates were washed four times with WB. Wells were blocked with 250 µl 1% (w/v) BB, sealed with acetate and incubated for 2 hr at RT (22 °C). Plates were washed twice with WB, air-dried for 10 min, then sealed with acetate and stored at 4 °C in an airtight container.

Assay procedure

BSA-MODMO Ag-coated plates were brought to RT (22 °C) for 1 hr prior to use. All reagents had been prepared in advance as previously described (5.2.1.4). Assays were conducted in quiescent solutions. Controls lacked Ag and/or biotinylated PAb.

4. *Biotinylated PAb incubation (with or without analyte)*: A 100 µl volume of analyte solution (containing MTBE, or a control solution containing no MTBE) was dispensed into plate wells immediately *before* the addition of 100 µl biotinylated PAb. Plates were sealed with acetate and incubated for 1 hr at RT. The plate was then washed four times with WB.
5. *Secondary incubation*: A 100 µl volume of s-HRP (diluted 1:5000) was added to each well. Plates were sealed with acetate and incubated for 30 min. followed by a further four wash steps.
6. *Colour development and detection*: Unless specified otherwise, 100 µl TMB (2.08 M) was added to each well and incubated in darkness for 10 min. at RT. The reaction was stopped by the addition of 100 µl Stop solution (2 M H₂SO₄). Absorbance (A₄₅₀) was read immediately.

5.2.1.5 Protocol 2: a further optimisation

5.2.1.5.1 Comparison of two s-HRP conjugates at Ag-coating concentrations of 250 & 500 ng/ml

Assays were performed using two batches of s-HRP conjugates from two independent suppliers; Dako and Prozyme (5.1.1.2). As the reagents were supplied at different concentrations, (0.63 mg/ml & 1 mg/ml respectively), these were both diluted to ~125 ng/ml. Assays were conducted on plates coated with two BSA-MODMO Ag concentrations (250 & 500 ng/ml, 1% w/v BB) to further confirm the optimal Ag-concentration, as selected from 5.2.1.3.6. Assays were otherwise conducted as described in Protocol 2 (5.2.1.4).

5.2.1.5.2 Detection of native (non-biotinylated) PAb using a secondary HRP-labelled antibody

Standard assays were run as Protocol 2, these acted as controls. In a second set of assays, native (non-biotinylated) PAb (1 mg/ml) was diluted 1:1000 (100 ng/well). The s-HRP was replaced with a secondary (2 mg/ml) anti-IgG mouse HRP-labelled IgG goat antibody (5.1.1.2). This was diluted to 1:4000 (50 ng/well). These assays were otherwise run as in Protocol 2.

5.2.1.5.3 Determination of primary (native) antibody & Ag concentrations

Assays were conducted to determine the optimal native PAb concentration for use with Ag-coated plates (5.2.1.4). Plates were prepared with two Ag-coating concentrations (250 & 500 ng/ml). Native PAb was tested at 500 & 1000 ng/ml concentrations. Plate C was a repeat of plate B. A 100 µl volume of antibody solution was added to an equal volume of MTBE solution in each well. A full range of MTBE concentrations (0-5000 ppm in PBS) were tested. The 2Ab-HRP was diluted 1:4000 (500 ng/ml). The assay was otherwise conducted as Protocol 2.

5.2.1.5.4 Comparison of native PAb and biotinylated antibody at reduced Ag-coatings (250 ng/ml)

For further confirmation of the improved assay performance when using native PAb (& 2Ab-HRP), assays were repeated using reduced Ag-coating concentrations (250 ng/ml). Assays using biotinylated PAb (& s-HRP) were also conducted. The concentration of native antibody (500 ng/ml) was half that of the biotinylated antibody. Antibody capture: 2Ab-HRP was used at 50 ng/well, the s-HRP concentration was at 12.6 ng/ml, as per the manufacturer's minimum specification. Controls lacked Ag. The assay was otherwise conducted as Protocol 2.

From results obtained in this and the previous Section, the Ag was kept at 500 ng/ml concentration to equal that of the native PAb primary and 2Ab-HRP concentrations for future assay development.

5.2.1.5.5 Non-specific binding of primary and secondary antibodies

NSB of the native PAb (500 ng/ml) and 2Ab-HRP (500 ng/ml) was investigated by comparing assays without MTBE with a number of controls. Plates were coated with Ag (500 ng/ml) and blocked with 1% BSA (w/v). Control 1 lacked PAb, Control 2 lacked 2Ab-HRP. Equivalent volumes (100 µl) of PBS was used in place of each reagent where required. The assay was otherwise conducted as Protocol 2.

5.2.1.5.6 Combined incubation of native PAb and 2Ab-HRP

Ag-coated plates (50 ng/well) were prepared and coated with an increased volume (300 µl) of 1% BSA (w/v). The incubation steps for the primary native antibody and the 2Ab-HRP were combined. Both PAb (100 µl) and 2Ab-HRP (100 µl) were added to MTBE solutions (0-5000 ppm in PBS, 100 µl/well) and incubated for 1 hr. The assay was then continued as Protocol 2. A standard assay (with two separate antibody incubations) was also performed. Controls lacked PAb.

5.2.1.5.7 Reduction of native PAb antibody incubation time

In a full-plate assay, the incubation of native PAb (with analyte) was reduced from 1 hr to 35 min. A second full-plate assay with a 1 hr incubation were conducted as a control. A new TMB substrate solution (Ultrasensitive TMB, 2.08 M) obtained from Europa Bioproducts Ltd., replaced the previous TMB solution (TMBUS, 5.1.1.1). Due to enhanced sensitivity, colour development was stopped after 3 min (instead of 10 min). Assays were otherwise conducted as Protocol 2.

5.2.1.6 ELISA Protocol 3

Protocol 3 is the result of further optimisation of Protocol 2 (5.2.1.4) with respect to the choice of PAb and secondary detection methods (5.2.1.5.2). Biotinylated PAb was replaced with native (non-biotinylated) PAb. A secondary HRP-labelled goat polyclonal anti-IgG mouse antibody was used to detect and label any bound native PAb in place of the s-HRP conjugate. A second series of experiments determined the optimal native PAb and 2Ab-HRP concentrations (5.2.1.5.3), Ag-coating concentration (5.2.1.5.4) and PAB–MTBE incubation times (5.2.1.5.6–5.2.1.5.7).

Assay performance had significantly improved overall using the native PAb/2Ab-HRP combination (both at 500 ng/ml concentrations) with respect to an increased dynamic range and reduced variation between replicates. Therefore, the native PAb (and 2Ab-HRP) was retained for all following assays. The ‘Ultrasensitive TMB’ (used in 5.2.1.5.7) gave a very rapid and improved colour response. Consequently, the colour development time was reduced from 10 to 3 min. With these improvements, the incubation time of PAb/analyte could be reduced from 1 hr to 35 min. without any loss in sensitivity. Table 5.4 gives a summary of the experimental conditions employed and retained for Protocol 3.

Table 5.4 Summary of optimisations retained for ELISA Protocol 3

The solutions were all prepared in PBS (10 mM, pH 7.4), 1 % BSA (w/v) was retained as the BB. Experimental parameters, conditions applied and retained are shown and cross-referenced to the appropriate Section.

Section	Parameters studied	Conditions applied	Conditions retained
5.2.1.5.2	Native PAb with 2Ab-HRP	<u>Dilutions:</u> PAB: 500 ng/ml 2Ab-HRP: 500 ng/ml	Native PAb: 500 ng/ml
	Biotinylated PAb with s-HRP	Biotinylated PAb: 1000 ng/ml s-HRP: 500 ng/ml	2Ab-HRP: 500 ng/ml
5.2.1.5.3	Ag concentration	250 & 500 ng/ml	500 ng/ml
5.2.1.5.4	Native PAb	500 & 1000 ng/ml	500 ng/ml
	2Ab-HRP	500 ng/ml	500 ng/ml
5.2.1.5.6	Incubation of native PAb & 2Ab-HRP	Native PAb incubation: 1 hr & 2Ab-HRP incubation: 30 min. Combined incubations: 1 hr	Separate incubations - PAb: 1 hr 2Ab-HRP: 30 min.
5.2.1.5.7	Incubation time of PAb (with analyte)	PAb incubations: 35 min. and 60 min.	PAb incubation: 35 min.
		Ultrasensitive TMB (2.08) used	Colour development reduced to 3 min. 2 M Stop solution (H ₂ SO ₄) retained

5.2.1.6.1 Repeatability of the MTBE ELISA using Protocol 3

To determine the repeatability and accuracy of the optimised MTBE ELISA, assays were performed on three Ag-coated blocked plates (500 ng/ml, 1% BSA w/v). The full range of spiked MTBE solutions were tested using Protocol 3 (5.2.1.6).

5.2.1.6.2 Effects of temperature, buffer choice and pH on assay response

5.2.1.6.2.1 Temperature

Assays were conducted at three temperatures: 4, 21 & 27 °C following Protocol 3 (5.2.1.6).

5.2.1.6.2.2 Buffer choice

Antibodies and MTBE solutions (0-5000 ppm) were prepared in three different buffers (10 mM, pH 7.4): PBS, PB, and Tris (5.1.1.1). Assays were conducted on three Ag-coated plates (500 ng/ml), following Protocol 3 (5.2.1.6).

5.2.1.6.2.3 Effect of pH on assay response

Antibodies and MTBE solutions (10, 30, 100 and 300 ppm) were prepared in different buffer standards (5.1.1.1); pH 4 (phthalate), 7 (phosphate) & 9.2 (borate). A full 96-well plate assay was performed for each pH following the assay procedure in 5.2.1.6. A second set of MTBE solutions were also made in local, unfiltered groundwater (pH 6.6). These were tested (with the antibodies diluted in PBS) following Protocol 3.

This concluded the developmental work for the 96-well ELISA. The following methods relate to further characterisation of the ELISA format and detection of MTBE and related organic compounds.

5.2.1.7 Stability of MTBE in buffer (spiked PBS)

5.2.1.7.1 MTBE volatility in PBS buffer

A glass Pasteur pipette was placed into a 25 ml polycarbonate tube containing a 10 ml solution of MTBE (5000 ppm) in PBS buffer (10 Mm, pH 7.4, 5.1.1.1) and held in place by a clamp. A second pipette was also placed into the solution for removing samples. A 0.5 ml volume was removed at the start of the experiment and immediately tested by ELISA using Protocol 3 (5.2.1.6). Nitrogen gas was then steadily bubbled through the MTBE solution. The MTBE solution was sampled (0.5 ml volumes) and tested by ELISA at 30 min intervals for a total of 4 hr. This experiment was performed twice. A standard full plate ELISA (Protocol 3) was run using fresh MTBE solutions (0-5000 ppm) in order to obtain a calibration curve.

5.2.1.7.2 Stability of MTBE solutions over time

Further experiments examined the stability of the MTBE solutions in PBS buffer (10 mM, pH 7.4). Solutions were prepared (0-5000 ppm) and stored at RT (22 °C). Assays were conducted periodically using these 'aged' MTBE solutions; after 3 hr, after 3 days and over a 3 week period (weekly testing). Fresh MTBE solutions were assayed on each occasion for comparison.

5.2.1.8 Detection of fuels and alternative oxygenates using the MTBE ELISA

MTBE solutions (0-5000 ppm) were prepared in PBS (5.1.1.3), as were other fuel oxygenate solutions; *tert* amyl methyl ether (TAME), ethyl *tert* butyl ether (ETBE), *di*-isopropyl ether (DIPE), *tert* butyl ether (TBA) & *tert* butyl formate (TBF), along with two oxygenate alternatives; ethanol (EtOH) and methanol (MeOH). Fuel mixtures (0-5000 ppm) were made using unleaded petrol & diesel (Section 3.1). These solutions were stirred (using a magnetic stirring bar) at RT (22 °C) for 1 hr prior to testing. Assays were performed as Protocol 3 (5.2.1.6).

5.2.1.9 Investigation of PAb binding characteristics with MODMO-structural analogues

5.2.1.9.1 Detection of MTBE & MODMO

Spectroscopic-grade MTBE and 7-methoxy-3,7-dimethyloctanal (MODMO) solutions (0-5000 ppm) were prepared (5.1.1.3). Two full-plate assays were performed using as Protocol 3 (5.2.1.6). Further serial dilutions (1:2 and 1:10 in PBS) from a single MODMO solution (5 ppm) were made and further assays conducted over this lower-end range.

5.2.1.9.2 PAb binding to short, straight chain alcohols (SCAs)

A group of straight-chain alcohols; 1-butanol, 1-propanol, 1-pentanol, 1-hexanol, 1-octanol and 2-propanol were prepared in PBS (5.1.1.3) with a concentration range between 0-10,000 ppm. MTBE solutions (0-10,000 ppm) were also assayed in the same plate for comparison. Assays were performed using Protocol 3 (5.2.1.6).

5.2.1.9.3 ELISA response to MODMO analogues (CH₃ group on 2-carbon)

The following compounds; 2-methyl, 1-butanol, 2-methyl, 1-propanol, 2-methyl, 1-pentanol can be viewed as SCAs with an additional, single methyl group (CH₃) bound to the 2nd carbon; these were assayed with MTBE as in 5.2.1.9.2.

A further set of assays examined alternative MODMO analogues; 2-methyl, 2- butanol; 2-methyl, 2-pentanol; 3-methyl, 1-butanol and 3-methyl, 1-pentanol. These structural analogues

varied in the position of the –OH group (1st or 2nd carbon) and the methyl group (2nd or 3rd carbon). Assays were performed as in 5.2.1.9.2.

5.2.1.10 Application & validation of the ELISA

5.2.1.10.1 ELISA performance using MTBE-spiked local groundwater

Local groundwater (1 l volume, pH 6.7) was collected in a sterile 1 l glass Duran from an on-site monitoring well. This was allowed to stand for 5 hr to allow any organic material (OM) to settle and to reach RT (22 °C). Two sets of serial dilutions were then made from a single 5000 ppm MTBE-spiked solution (1:2 and 1:10 respectively). Assays were then conducted as Protocol 3 (5.2.1.6).

A further series of experiments were conducted to compare assay response in buffered and non-buffered groundwater. Local groundwater (pH 6.7) was collected in two sterile 1 l glass Durans from an on-site monitoring well (5.1.1.4). These were then filtered through 12.5 cm dia. Whatman 114 (190 µm thickness, 25 µm exclusion) filter papers and left overnight. Unfiltered (1 l) groundwater was buffered with PB (10 mM, pH 7.4, 5.1.1.1). A 20 ml volume of each groundwater was then spiked with MTBE (5000 ppm) and serial dilutions made (0-5000 ppm) in 20 ml volumes of the same water. A range of MTBE solutions (0-5000 ppm) in PBS (10 mM, pH 7.4) prepared 48 hr previously were also assayed.

5.2.1.10.2 ELISA performance using MTBE-spiked Austrian groundwater

Two groundwater samples (obtained from Salzburg, Austria, 5.1.1.4) were spiked with MTBE and assayed. The two groundwaters ('Moorwasser' & 'Fürstenbrunn') had very different properties, summarised in Table 5.5. These had been stored at –20 °C for 3 months prior to testing. Each was thawed, brought to RT (22° C) and a 20 ml volume of each was spiked with MTBE (5000 ppm). Serial dilutions (1/10) were then made using the same groundwater (0-5000 ppm). Further dilutions were made from both 50 ppm groundwater solutions (5-50 ppm). MTBE-spiked PBS solutions were also prepared (10 mM, pH 7.4). Assays were then conducted following Protocol 3 (5.2.1.6).

GC/MS validation of the ELISA

Further 100 ml volumes of each groundwater (Table 5.5) were then spiked with MTBE (10, 25, 35, 40 & 60 ppm). These were then divided into glass GC/MS sampling vials (two per concentration), filled to the top (42 ml) and sealed. After 8 days, one set of samples were assayed by ELISA, the other set of spiked samples were independently analysed by GC/MS, as follows:

Table 5.5 Austrian groundwater physico-chemical properties

Summarised water analysis data of the two groundwater samples, Quelle Fürstenbrunn (Groundwater A) and Moorwasser (Groundwater B) are shown. Full water analysis details are provided in Appendix 4.

Water analysis	Groundwater A	Groundwater B	~ % ratio
Water site name	Quelle Fürstenbrunn	Moorwasser (Leopoldskroner moor)	
Quality	Clear	Brown, particulate	
pH	7.77	6.97	
Turbidity (FTU) *	1.18	8.55	14:86
Total Cations (mg/l)	33.82	126.56	26:74
Total Anions(mg/l)	5.44	36.32	15:85
TOC	2.87	10.68	27:73

* ISO Standard - Formazine Turbidity Unit (FTU)

ELISA methodology

One set of the MTBE-spiked Austrian groundwater samples were diluted (1:1) and assayed on the same plate as a range of MTBE solutions (0-500 ppm) following Protocol 3. This was tested twice on two separate microtitre plates. The MTBE results were plotted together for a calibration curve against which all groundwater results were determined. Sample concentrations were then multiplied x 2 to account for the dilution factor.

Methodology for Purge & Trap - GC/MS analysis

The other set of the MTBE-spiked Austrian groundwater samples were analysed by Purge & Trap GC/MS (P&T-GC/MS) using a SOLATek 72 Multi-Matrix Vial Autosampler. Quantification was performed using Xcalibur Quantitative Analysis by Internal Standard Calibration (Fluorobenzene).

Each sample was manually diluted by injecting 100 µl of sample (using a volumetric syringe) into 42 ml HPLC grade water contained in sealed glass GC/MS vials. Three duplicates were made for each spiked sample. Preliminary P&T-GC/MS analysis used a tentative dilution factor of 1:50 (automated dilution) so to avoid potential contamination of the system. Results revealed that these samples were too dilute, and so the remaining two duplicates (per sample) were diluted 1:20 and analysed. Results were obtained from these two replicates only.

5.2.1.10.3 Analysis of natural, MTBE-contaminated groundwater samples (from Dresden, Germany)

MTBE and BTEX-contaminated groundwater was obtained from Dresden, Germany (5.1.1.4). Samples had been obtained via underwater motor pumps from 3 wells 200 m downstream of a MTBE refinery site in Leuna, Dresden. Samples were transported (~1 week) in sealed 20 ml GC/MS vials, preserved with sodium azide (0.1%) and were assayed by ELISA immediately upon receipt. Each sample was diluted in RO water (1:2) across the plate wells to a final dilution of 1:32. Samples were tested in conjunction with a range of MTBE solutions (0-5000 ppb, in PBS (5.1.1.1)) following Protocol 3 (5.2.1.6). Reading from the calibration curve and limiting the absorbance range to between 0.7-1.5 (A450), which equated to 5-500 ppm, determined MTBE concentrations. Dresden University tested duplicate samples by GC-FID headspace analysis.

5.2.1.10.4 Field testing & 'blind trial' validation of the MTBE ELISA (Koblenz, Germany)

The MTBE ELISA application at the SENSPOL Technical Meeting, Koblenz, Germany (5.1.1.4) was executed in a three-step approach:

1. Multiple water samples were collected (in 500 ml plastic containers) from various sites during the SENSPOL meeting and spiked with MTBE and BTEX compounds to levels determined by Tübingen University, Koblenz. The contents and concentrations were known only to Tübingen University and were withheld from trial participants.
2. The MTBE ELISA was conducted on-site (5.2.1.10.4.1) using one set of samples by the WATCH project co-ordinator, a geologist who had received only brief training in ELISA techniques.
3. Identical samples were forwarded to three groups within the WATCH project: consortium for independent 'blind trial' assay validation. These were; Cranfield University (ELISA), Diaclone SAS (ELISA) and GC/MS by Consejo Superior de Investigaciones Científicas (CSIC, Barcelona, Spain).

5.2.1.10.4.1 On-site application at Koblenz, Germany (Protocol 1)

Two complete MTBE ELISA test kits, each including a 96 well microplate, were supplied and prepared for application by Diaclone SAS (5.1.1.2), so that two field tests were possible. Five samples were tested by serial dilution (1:2) across the wells. Dilutions were made in RO water (pH unknown). In contrast to the Cranfield ELISAs (Protocol 3), coated plates (250 ng/ml Ag) were used in conjunction with biotinylated PAb & s-HRP. An unknown brand (of unknown

concentration) of TMB was used, with a 5 min colour development time. The assay was otherwise conducted as Protocol 1 (5.2.1.2). Results were then compared with a range of MTBE solutions (0-220 ppm in PBS), which had been also assayed as controls.

5.2.1.10.4.2 Independent analysis (ELISA) & P&T-GC/MS validation

Assays were conducted 1 week after the field trials by Diaclone SAS, following the same procedures described in 5.2.1.10.4.1. Independent P&T-GC/MS analysis of the Diaclone SAS samples was performed by CSIC.

5.2.1.10.4.3 Blind trial analysis (multiple ELISA) at Cranfield and independent Purge &Trap GC/MS validation

Duplicate river water samples (from Koblenz), contaminated with unknown quantities of MTBE & BTEX were independently assayed at Cranfield University (by multiple ELISA). Duplicate Cranfield samples were also tested by CSIC (Purge &Trap GC/MS). This was done to validate the novel ELISA by way of comparison with a classic analytical technique.

Immediately on receipt of the five bottled (300 ml) groundwater samples, six aliquots (42 ml) were made from each sample into sterile glass GC/MS vials. Two aliquots of each sample were firstly passed through a 5 µm Sartorius filter. Two unfiltered aliquots (42 ml from each sample) were immediately forwarded to CSIC (Barcelona, Spain) for independent GC/MS analysis. One aliquot of each Cranfield sample (filtered and unfiltered) was stored at 4 °C for testing in co-ordination with the Purge & Trap-GC/MS analysis after 1 week. The remaining samples were stored at -20 °C.

Multiple, independent full-plate assays were performed on seven individual Ag-coated plates following Protocol 3, except the TMB solution used was supplied by Moss (2.08 mM, 5.1.1.1). Duplicate serial dilutions (1:2) for each water sample were made across plate wells to a final dilution of 1:128. MTBE solutions (0-5000 ppm) were prepared in PBS (5.1.1.3) and assayed on the same plate (across 16 wells) each time for calibration. This equated to using 16 wells per unknown water sample per plate. This was repeated seven times.

MTBE concentrations were then determined from this (by reading each plotted point off the x-axis) and then multiplying by the dilution factor. It was found that limiting the absorbance range to the more linear part of the calibration curve (0.6-1.5) was necessary, equating to a MTBE concentration range of ~5-500 ppm.

Two further full-plate assays were also performed to examine the effects of filtration. Filtered samples (5 μ m) and unfiltered samples were assayed following Protocol 3 (5.2.1.6) and compared.

Unfiltered samples were also tested for BTEX compounds using the Total BTEX RaPID immunoassay test kit (Section 1.5.2.2), following the manufacturer's protocol (Appendix 3). GC/MS analysis for BTEX was also conducted by CSIC (Barcelona, Spain).

5.2.2 Protein conjugations

5.2.2.1 Determination of the MODMO carboxyl group activity

The activity of the MODMO aldehyde group was determined using a Pierce Glycoprotein Detection Reagent (5.1.2.2). All reagents were brought to RT (22 °C) and freshly prepared prior to use.

Reagent preparation: Sodium *meta*-periodate was prepared by dissolving 10.7 mg of NaIO₄ in 5 ml sodium phosphate buffer (10 mM, pH 6.3) and a 0.5% solution of glycoprotein detection reagent (GDR) was made by dissolving 5 mg GDR in 1 ml sodium hydroxide (1 M). Controls lacked MODMO (aldehyde groups) and MODMO/GDR.

Assay protocol: Equal volumes (50 μ l) of MODMO and sodium phosphate were placed into microtitre wells (n=6). For the oxidation step, 25 μ l of NaIO₄ was immediately added. This was replaced with 25 μ l buffer for the unoxidised test. The plate was shaken for 30 s on a plate shaker and then incubated at RT for 10 min. A 150 μ l volume of GDR was added to each well and the plate shaken for a further 30 s. The plate was then incubated for 1 hr and the absorbance read at 550 nm.

5.2.2.2 Conjugation of MODMO-HRP

Immediately prior to the conjugation procedure, free HRP (4 mg) was dissolved in 1 ml borate buffer. The reducing agent NaCNBH₃ (10 mg/ml) was also freshly prepared in borate buffer. MODMO (20 μ l) was diluted in 80 μ l DMF. As the conjugation method was developed throughout this work, minor variations in the conjugation methods are detailed in Table 5.6. Each compound was added to 2 ml Eppendorfs in the concentrations and order shown. Removal of unconjugated MODMO & HRP was by dialysis (5.2.2.2.1).

Table 5.6 Methods for conjugation of MODMO to free HRP

The following Table shows the method development and variations employed for the MODMO-HRP direct conjugations. Each solution was applied in the order shown. The conjugation protocol shown was used for all 4 methods.

Method	1	2	3	4
Conjugation mix & order	100 µl MODMO (10 mM in DMF)	500 µl HRP * (4 mg/ml)	500 µl HRP (4 mg/ml)	500 µl HRP (4 mg/ml)
	500 µl HRP (4 mg/ml)	100 µl MODMO (10 mM in DMF)	100 µl MODMO (100 mM in DMF)	500 µl NaCNBH ₃ (100 mg/ml)
	500 µl NaCNBH ₃ (10 mg/ml)	500 µl NaCNBH ₃ (10 mg/ml)	500 µl NaCNBH ₃ (10 mg/ml)	100 µl MODMO (10 mM in DMF)
Borate buffer pH	7.7	7.7	9.2	9.2
M conc.	0.2	0.2	0.1	0.1
Conjugation	3 hr slow rotation at RT (22 °C) & 18 hr slow rotation at 4 °C			

5.2.2.2.1 Dialysis protocol

The conjugated solutions were purified using Pierce ‘Slide-A-Lyzer’ dialysis cassettes & accessories (5.1.2.2). Cassettes were rehydrated for 30 s in PB (5.1.2.1) prior to use. Excess liquid was removed by gentle tapping on paper towels. Conjugated solutions were carefully injected into the cassette and any air was withdrawn using the syringe. The cassette was then attached to a buoy and placed into 5 l PB buffer. Cassettes were very slowly rotated using a magnetic stirrer. Dialysis was performed three times in fresh 5 l volumes of PB, twice for 2 hr at RT (22 °C) and finally over 18 hr at 4 °C. This dialysis protocol was used for all conjugate purification. Purified conjugate was stored at –20 °C (100 µl aliquots) and thawed immediately prior to use.

5.2.2.3 General assay procedure for testing prepared MODMO-HRP conjugates

5.2.2.3.1 Microtitre plate preparation (PAb coating)

Nunc™ microtitre plates (5.1.1.4) were coated with 100 µl native PAb (5.1.1.2) diluted in PBS to either 500 or 1000 ng/ml. Plates were coated overnight at 4 °C, washed with WB (x 4) and air-dried. One set of plates was blocked with 300 µl (per well) of 1% BSA (w/v) for 2 hr at RT. Plates were then washed twice with WB and air-dried. Plates were stored at 4 °C and brought to RT prior to use. Another set of plates were left unblocked, these were used immediately.

5.2.2.3.2 General assay procedure

The stored MODMO-HRP conjugate was thawed at RT (22 °C) prior to use and then diluted to 500 & 1000 ng/ml in PBS (5.1.2.1). A 100 µl volume of each MODMO-HRP concentration was

added to prepared PAb-coated microtitre plates (5.2.2.3.1) and incubated at RT for 1 hr. A stock solution of free HRP was made by dissolving 2 mg HRP in 1.4 ml borate buffer (0.2 M, pH 7.7), which was then diluted to 1000 ng/ml (7 µl stock in 10 ml borate buffer). This was tested (100 µl/well volume) in the most concentrated antibody-coated wells (1000 ng/ml) as a NSB control. Post incubation, four washes were performed with WB (5.1.2.3). A 100 µl volume of TMB was added to the wells and incubated at RT for 20 min. Colour development was stopped using 100 µl Stop solution (2M H₂SO₄) and the absorbance read at 450 nm.

5.2.2.3.3 Assays testing MODMO-HRP at two temperatures

MODMO-HRP conjugates (prepared using conjugation methods 3 & 4, Table 5.6) were tested at 22 & 28 °C. In contrast to the general assay procedure (5.2.2.3), the amount of conjugate per well was doubled to 200 µl. Plates were incubated at the two temperatures for 1 hr. The colour development step was as 5.2.2.3.

5.2.2.4 Conjugation of BSA-MODMO & BSA-MODMO-HRP

5.2.2.4.1 Conjugation of BSA-MODMO (antigen preparation)

The conjugation of BSA-MODMO antigen (as supplied by Diaclone SAS, 5.1.1.2) was made using Maxibind™ cationised BSA (5.1.2.2) following the manufacturer's supplied protocol (Uptima/Interchim, France), as follows:

The Maxibind™ BSA (5 mg) was dissolved in 1 ml tetraborate buffer (0.05 M, pH 9.8). MODMO (20 µl) was mixed with DMF (80 µl) to give a 1 M solution. A fresh solution of NaCNBH₃ was prepared by dissolving 0.5 mg in 1 ml NaOH (1 M). The MODMO/DMF was then slowly added to the BSA solution, immediately followed by 10 µl of NaCNBH₃ solution. The conjugation mixture was incubated for 4 hr (RT) on a Dynal sample mixer (5.1.2.2). Unbound reagents were removed by dialysis (5.1.2.2).

5.2.2.4.2 Conjugation of BSA-MODMO-HRP complex

The BSA-MODMO-HRP conjugate (5.1.2.2) tested was supplied by Diaclone SAS after following two basic coupling procedures supplied by the BSA manufacturer (Uptima/Interchim). However, a proprietary enzyme amplification procedure was reported to have been used and so the exact conjugation procedure cannot be described. The supplied BSA-MODMO-HRP conjugate was diluted in PBS and tested following the MODMO-HRP procedure (5.2.2.3).

5.2.2.5 General assay procedure for testing prepared BSA-MODMO-HRP conjugates

Microtitre plates were prepared as 5.2.2.3.1. Assays using the BSA-MODMO-HRP conjugate on blocked and unblocked plates were conducted as for the MODMO-HRP conjugate (5.2.2.3.2). The BSA-MODMO-HRP merely replaced the MODMO-HRP conjugate.

5.2.2.5.1 Checkerboard assay with the BSA-MODMO-HRP conjugate

Native PAb was coated across microtitre wells in serial dilutions (1:2 in PBS) from 125-1000 ng/ml and blocked (5.2.2.3.1). BSA-MODMO-HRP conjugate was serially diluted (1:2 in PBS) from 125-1000 ng/ml, with 100 µl added across the coated wells and incubated at RT (22 °C) for 1 hr. Free HRP was diluted to 1000 ng/ml. A 100 µl volume (per well) was tested on the most concentrated antibody-coated wells (1000 ng/ml) as a NSB control. Plates were washed with WB (x 4). TMB (100 µl) was added to each well and left for 20 min and then stopped with 100 µl Stop solution (2 M H₂SO₄) and the absorbance measured at 450 nm. NSB controls were bare wells.

5.2.2.6 Differentiation of MTBE solutions by MODMO-HRP & BSA-MODMO-HRP conjugates

Both conjugates were tested with a range of MTBE concentrations. MTBE solutions (50, 500 & 5000 ppm) were freshly prepared in PBS prior to use. Microtitre plates were coated with PAb (500 ng/ml) and blocked with 1% BSA (w/v, 5.2.2.3.1). Each MTBE solution was then added to wells (100 µl) followed by 100 µl of each conjugate (1000 ng/ml). Plates were incubated for 1 hr (RT). Washing steps and colour development were as 5.2.2.3.2.

5.2.2.7 Orientation of the PAb

In order to ensure correct orientation of the PAb, it was immobilised on Pierce 'Reacti-bind' goat anti-IgG mouse pre-coated antibody plates (5.1.2.3). The response was compared with a standard (Nunc) plate, which had no pre-coated anti IgG antibody. Both plates were incubated with two PAb concentrations (500 & 1000 ng/ml) and 48 of 96 wells were blocked with 1 % BSA (w/v). The procedure was otherwise as 5.2.2.3.1. Assays were then conducted as 5.2.2.3.2 using both MODMO-HRP and BSA-MODMO-HRP conjugates. Free HRP (1000 ng/ml) was used as a control.

5.2.3 ELISA Methods

All assays were conducted at RT (22 °C). Petri dish membrane incubations were conducted on a plate shaker (5.1.3), unless otherwise specified.

5.2.3.1 Pre-coating membranes with Ag

Membranes (6 mm discs) were coated with Ag (1000 ng/ml) by pipetting 5 µl of Ag solution onto the membrane surface, which was enough to evenly coat the membrane surface and calculated at 47 ng per membrane. Membranes were then air-dried for 15 min. Blocking steps are reported in each appropriate methods section.

5.2.3.2 Membrane selection

5.2.3.2.1 Blocking buffer efficiency against NSB

The effectiveness of three different blockers - 2% BSA (w/v), 1% (v/v) Casein in TBS and Superblock (undiluted) against NSB was evaluated. Membranes (5.1.3) were tested without PAb through steps 1-4, as follows: (in a repeat set of experiments, the final washing step (post-HRP incubation) was extended from 10 to 30 min.).

1. *Blocking step:* Ag-coated membranes (5.2.3.1) were blocked with each one of the BBs (2 ml) in 2.5 ml Eppendorfs, which were gently rotated for 1 hr.
2. *Wash step:* The membranes were taken out of the respective Eppendorfs, excess BB was removed by washing each set of blocked membranes (BSA, Casein or Superblock) in a glass beaker containing PBS (40 ml), which was gently stirred (using a magnetic stirring bar) for 10 min. Membranes were then placed on paper towels, any excess surface liquid was removed by gently blotting with tissue paper and membranes were left to dry for 15 min.
3. *HRP-labelling steps:* s-HRP (200 ng/ml) and 2Ab-HRP (500 ng/ml) were prepared in 20 ml PBS. Each set of blocked membranes were then placed into individual Petri dishes containing 4 ml of either s-HRP or 2Ab-HRP solution, covered and gently shaken for 30 min.
4. *Wash steps:* Excess reagent was removed by two further washing steps in PBS (40 ml), each for 10 min. Excess liquid was again removed by gently blotting with tissue paper (as step 2).
5. *Colour development:* A 5 µl volume of TMB (Sigma, 5.1.3) was then pipetted onto the centre of each membrane and left for 10 min. Any colour development was visually assessed & recorded after 1, 2, 5 & 10 min.

5.2.3.2.2 Ag-PAb binding on membrane surfaces

In a further series of experiments, assays were conducted with and without a PAb incubation step. Assays were conducted as in 5.2.3.2.1, except a PAb incubation step was added between steps 2 & 3.

PAb incubation: Each set of blocked membranes (1% w/v BSA, 1% Casein or Superblock) were placed in individual Petri dishes containing 4 ml of either biotinylated PAb or native PAb solutions (100 ng/ml) and shaken for 30 min. Where no PAb was used, membranes were incubated in an equal volume of PBS.

The assays were then continued following steps 3-5 in 5.2.3.2.1. However, in these experiments, Tween 20 (0.01%) was added to the final PBS wash step (step 4, 5.2.3.2.1).

5.2.3.3 Complete assays conducted on membrane surfaces in Petri dishes

The next step was to see if MTBE could be detected by running full assays on the membrane surface. In contrast to the previous experiments, membranes were coated with Ag by placing the membranes in a Petri dish containing 4 ml Ag solution (1000 ng/ml), which was gently shaken for 1 hr. Membranes were then blocked by placing each set of membranes in 4 ml of either BB (1% w/v BSA, 1% Casein or undiluted SB) in Petri dishes, shaken for 10 min and then washed as in step 2, 5.2.3.2.1.

Two sets of each membrane/BB combination were placed into individual, Petri dishes containing 2 ml of either biotinylated PAb, native PAb or PBS. A 2 ml volume of MTBE (5000 ppm in PBS) was then added to one set, and PBS (2 ml) added to the remaining set. The PAb incubation was for 30 min. A schematic of experimental parameters is shown in Fig. 5.1.

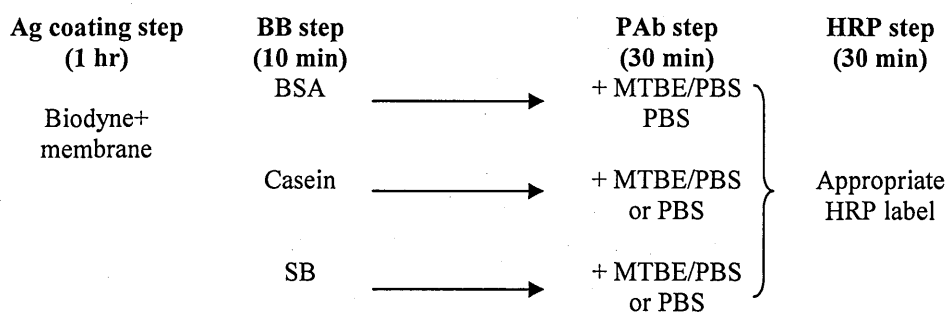


Fig. 5.1 Schematic of experimental parameters for membrane assays

Experimental design for testing Biodyne+ membrane with or without MTBE present during the PAb binding step. This was tested using both biotinylated and native PAb with the appropriate HRP label (s-HRP or 2Ab-HRP). Immunodyne & Hybond+Nylon membranes were also tested in turn.

After PAb binding (with or without MTBE), membranes were removed and incubated with 4 ml of the appropriate HRP labelling reagent (s-HRP or 2Ab-HRP) and shaken for 30 min. Two wash steps were then performed (as Step 4, 5.2.3.2.1). TMB (5 μ l) was pipetted onto the centre of each membrane, visual assessment of colour development on the membranes was recorded and compared after 2 min.

5.2.3.4 Evaluation of membranes using ELIFA Protocol 1

The transposing of the ELISA into an ELIFA format used the experimental set-up described in 1.6.5. Pilot ELIFA experimental methodology was initially based on that employed for the ELISA. A single MTBE solution (5000 ppm) was prepared in PBS (5.1.3) and premixed in equal volumes (100 μ l) with the native PAb (1:1) and used as a positive control. This was tested against PAb/PBS mixtures (1:1) containing no MTBE.

The preliminary flow-through volumes of reagents and order of application for Protocol 1 was based on that used for the ELISA (Table 5.7). A membrane was placed in the flow cell and the two components were clamped together. Membranes were pre-wetted by pipetting PBS (600 μ l) into the loading chamber, which was pulled through the membrane by a peristaltic pump (5.1.3) with a flow rate of 0.4 ml/min. The other reagents were pipetted into the loading chamber after the preceding reagent had almost passed completely through the membrane. At no time was the membrane allowed to dry out, as this would have created an air bubble and prevented constant flow. Washing steps for the ELIFA used 3 x 300 μ l volumes of WB (0.01% v/v Tween 20 in RO water).

Finally, TMB solution was pumped through and held in the chamber (by ceasing pumping). Colour development was stopped by adding Stop solution (1 M H₂SO₄) into the loading chamber. As these two steps were varied during the ELIFA method development, flow rates, holding times and other variations are described for each appropriate protocol.

At the end of the ELIFA, the coloured solution was extracted from the loading chamber by disconnecting the tubing and pulling the solution (held in the flow cell) through the bottom chamber by pumping through the cannula. A 200 μ l volume of collected solution was transferred to a microtitre plate well and the absorbance read at 4450.

Table 5.7 Volume and order of reagent addition for the ELIFA Protocol 1

Protocol 1 was applied to all membranes (5.1.3). ELIFAs were conducted using each membrane with all BBs (BSA, Caseine or Superblock, 5.1.3). This was conducted using biotinylated PAb/s-HRP and then native PAb/2AB-HRP combinations.

Protocol 1		
Reagents	Volume [μ l]	Flow [ml/min] ¹
Pre-wetting	600	0.4
Ag	100	0.04
BB ²	250 (1%)	0.04
Wash step	900	0.4
PAb/PBS or PAb/MTBE ³	200	0.04
HRP ⁴	100	0.02
Wash step	900	0.4
TMB	200	0.04/30 s Hold 30 s
Stop	200	N/A
Absorbance volume (A450)	200	N/A

¹ Flow rate was ml/min unless contra-indicated in brackets.

² BSA (1% w/v), Superblock or Caseine (1% w/v)

³ Either biotinylated or native PAb

⁴ Either s-HRP or 2Ab-HRP. N/A – not applicable.

5.2.3.5 Evaluation of Immunodyne membrane & further optimisations

Evaluation of the Immunodyne membrane was made using a revised protocol (Protocol 2). Membranes were pre-coated with Ag (as 5.2.3.1). This protocol was then optimised by changing a number of factors (Table 5.8) which led to Protocol 3 and finally Protocol 4. Results were compared with ELISAs conducted on Ag-coated microtitre plates (Protocol 2, 5.2.1.4) using the same reagent mixtures (PAb, s-HRP) used for the ELIFA.

5.2.3.5.1 Protocol 5 – further optimisations

In Protocol 5, the PAb (100 ng/ml) was pre-incubated with free MTBE prior to running the ELIFA. Equal 100 μ l volumes of PAb and MTBE (5000 ppm) were mixed in an 0.5 ml Eppendorf, gently mixed for 30 s and stored at 4 °C for 1 hr prior to use in the ELIFA. Additional modifications to Protocol 4 were:

1. Wash buffer volumes were reduced from 900 to 600 μ l.
2. A constant flow rate of 0.130 ml/min was used throughout the ELIFA.
3. The volume of TMB was reduced from 200 to 150 μ l. Colour development was stopped with 50 μ l of 2 M Stop solution (instead of 200 μ l of 1 M).

The assay was otherwise run as Protocol 4 (Table 5.8). Results were again compared with ELISAs conducted on Ag-coated microtitre plates (Protocol 2, 5.2.1.4) using the same reagent mixtures (PAb, s-HRP) used for the ELIFA.

Table 5.8 ELIFA protocols used for evaluation of Immunodyne membranes

The order and quantity of each reagent used for Protocols 2-4 are shown. Reagents are listed in the first column. BSA was used as the BB throughout – % w/v concentrations are indicated.

Protocol 2	Colour		Protocol 3	No colour	Protocol 4	Colour
Reagents	Volume [μl]	Flow [ml/min]	Volume [μl]	Flow [ml/min]	Volume [μl]	Flow [ml/min]
Pre-coated Ag (1000 ng/ml)	5	N/A	5 (1:10 dilution)	N/A	5 (1:10 dilution)	N/A
BB (% BSA w/v)	None	None	200 (1%)	0.08	200 (0.1%)	0.04
Wash step	900 (PBS)	0.13	900	0.13	600	0.4
PAb/PBS or PAb/MTBE ¹	200	0.02	200	0.04	200	0.04
HRP ²	100	0.02	100	0.04	100	0.04
Wash step	900	0.4	900	0.13	900	0.4
TMB	200	0.04/30 s Hold 60s Reverse 0.04/30 s	200	0.04/30 s Hold 60 s Reverse 0.04/30 s	200	0.04/30 s Hold 5 min. Reverse 0.04/30 s
Stop	200	N/A	200	None	100	N/A
Absorbance vol. (A450)	200	N/A	200	None	200	N/A

¹ Either biotinylated or native PAb ² Either s-HRP or 2Ab-HRP. N/A – not applicable.

5.2.3.6 Evaluation of Biodyne+ membrane

ELIFAs were firstly conducted using Ag-coated Biodyne+ membrane, following Protocol 5 (5.2.3.5.1).

5.2.3.6.1 Protocol 6; optimised Biodyne+ ELIFA

Protocol 5 was again modified to optimise ELIFA performance on the Biodyne+ membrane, leading to Protocol 6 (Table 5.9). This protocol was then used to evaluate two BBs, previously shown to be most effective (5.2.3.2.1) with this membrane; 1% (w/v) BSA and undiluted SB.

5.2.3.6.2 Evaluation of Biodyne+ Protocol 6 with native PAb/2Ab-HRP

The biotinylated PAb/s-HRP was then replaced with native PAb/2Ab-HRP (5.1.3) and further ELIFAs were conducted following Protocol 6 (Table 5.9). However, BSA blocker was replaced with Superblock.

Further ELIFAs were then conducted as 5.2.3.5.2 using the native PAb/2Ab-HRP combination. In these assays, the BB was replaced with Casein (1% w/v in TBS), which was further diluted 1:10 in RO water to 0.1% w/v). This then concluded the development work for the ELIFA.

Table 5.9 Protocol 6 for ELIFA with Biotyne+ membrane

This protocol was developed for ELIFA using biotinylated PAb/s-HRP specifically on the Biotyne+ membrane. In contrast to Protocol 5, the Ag was immobilised onto the membrane by slowly pumping the Ag solution through.

Protocol 6		
Reagents	Volume [μl]	Flow [ml/min]
Ag (1000 ng/ml)	100	0.05
BB ¹	0.05	0.05
Wash step	600	0.1
PAb/PBS or PAb/MTBE ²	200	0.10
HRP ³	100	0.05
Wash step	900	0.10
TMB	150	0.05/30 s Hold 5 min Reverse 0.05/30 s
Stop	50	N/A
Absorbance vol. (A450)	200	N/A

¹ Both BSA (1% w/v) and Superblock (undiluted) were tested.

² Either biotinylated or native PAb

³ Either s-HRP or 2Ab-HRP. N/A – not applicable.

5.2.4 Magnetic bead immunoassay

5.2.4.1 Introduction

The Dynabeads were supplied (2×10^9 or ~30 mg/ml bead concentration) in a 1 ml preservative solution. Prior to use, these required resuspension followed by a washing step. The beads then had to be coated with the appropriate reagent, in this case Ag, followed by blocking and washing steps. The beads were then resuspended in a storage buffer (Buffer C) prior to use in immunoassay. These steps are described in the following Sections.

Once the beads had been coated with Ag, a novel magnetic bead-based immunoassay was developed for MTBE detection using Tosylactivated magnetic beads (T-beads). The method development and subsequent test assays are described in the following Sections.

5.2.4.2 Coating Tosylactivated Dynabeads® (T-beads) with Ag

1. *Bead resuspension & washing step*

T-beads were first resuspended in their supplied preservative solution by alternating gentle pipetting & vortexing for 1 min. The beads were then transferred into a 2.5 ml Eppendorf tube and placed onto the magnetic rack (Chapter 1, Fig.1.14). This draws & holds the magnetic beads to the side of the tube facing the magnet. After 5 min, the supernatant was carefully removed by pipette, leaving the beads in place.

The tube was then removed and the beads were washed in 1 ml buffer A (phosphate, 5.1.4.1) by gentle mixing on the tilt rotator (10 rpm) for 2 min. The tube was then placed back into the magnetic rack for 5 min. and the buffer then aspirated by pipette. The beads were then resuspended in 500µl buffer A (equating to a x 2 bead concentration of 4×10^9 or ~ 60 mg/ml)

2. *Ag-coating*

A 450 µl volume of Ag (106 µg/ml) was diluted in 180 µl buffer A in a 2.5 ml Eppendorf tube and 370 µl washed beads added to give a 1 ml coating solution containing 48 µg Ag and 1.48×10^9 beads. After a light 30 s vortex, the tube was placed on the rotator and gently mixed at 8 rpm for 48 hr at RT (22 °C).

3. *Washing steps*

The tube was placed into the magnetic rack for 5 min and the supernatant removed. The tube was removed and beads were washed by adding 1 ml buffer C (PBS/BSA, 5.1.4.1), which was at 4 °C and mixed on the rotator for 5 min. The tube was placed on the magnetic rack for 5 min and then aspirated by pipette. This wash step was then repeated.

4. *Blocking steps*

The Ag-coated beads were then blocked with 1 ml buffer D (Tris/BSA, 5.1.4.1) over 24 hr on the rotator at RT (the Tris/BSA buffer blocked any remaining free Tosyl-activated groups on the magnetic beads). The tube was then placed onto the magnetic rack for 5 min. and the buffer aspirated. The tube was removed and the coated & blocked beads were then resuspended in 1 ml buffer C (Ag coated at 48 µg/ml on 1.48×10^9 beads/ml) and stored at 4 °C prior to use in the following immunoassays.

5.2.4.3 Immunoassay development (magnetic bead format) – Protocol 1

Preparation of stock immunoreagent solutions

Stock solutions of PAb & 2Ab-HRP were made to a concentration of 1 µg/ml in buffer A. A further 1:10 dilution of 2Ab-HRP was made in buffer A to 100 ng/ml. Stored beads were resuspended by gentle vortexing and diluted 1:100 in buffer A (to 1.48×10^7 bead/ml stock solution).

5.2.4.3.1 Magnetic bead immunoassay

The assays were conducted following steps 1-4, as follows:

1. PAb incubation

In 2.5 ml Eppendorf tubes, 400 µl buffer A was mixed with 400 µl sample (either PBS or concentrations of MTBE in PBS – see individual experiments). Equal 100 µl volumes of PAb & beads were then added to each tube, giving a final assay concentration of 100 ng PAb: 1.48×10^6 beads in a 1 ml volume. Tubes were sealed, inverted x 3 and placed on the rotator for 30 min. After this time, tubes were transferred to the magnetic rack and left 2 min for the beads to settle.

2. Assay wash steps

Three wash steps were performed in the magnetic rack using 1 ml volumes of WB (buffer C at 4 ° C). The supernatant was removed by pipette. The magnetic strip was removed (therefore leaving the tubes in the rack) and 1 ml WB (buffer C) added to each tube. The tubes were sealed and the rack inverted x 3 to mix. This was then left for 2 min. The magnet was replaced, left for 2 min. and the WB aspirated and the wash steps repeated twice. Tubes were then removed from the rack.

3. 2Ab-HRP incubation

1 ml volumes of the 2Ab-HRP stock solution (100 ng/ml) were added to each tube, which were sealed and placed on the rotator for 30 min at RT. Tubes were then transferred to the magnetic rack and left for 2 min. Assay wash steps were repeated.

4. Colour development

After the final wash step, the colour substrate solution (TMB, 500 µl) was added to each tube. The magnetic strip was removed and the rack (& tubes) was manually inverted (x 3) to mix and then left for 10 min. at RT for the colour to develop. The reaction was then stopped with an equal volume of Stop solution (1 M H₂SO₄). The magnetic strip was then replaced to remove

the beads from solution. After 1 min, three aliquots of reactant (3 x 300 µl volumes from each assay tube) were transferred to a microtitre plate and the absorbance read at 450 nm. The average reading (n=3) was then taken for each assay.

5.2.4.4 Preliminary magnetic bead based assays (Protocol 1)

5.2.4.4.1 NSB of 2Ab-HRP

Two separate tests were performed (6 assays per test) to evaluate the degree of NSB from the 2Ab-HRP. PBS buffer (400 µl) was used as a 'sample'. PAb was replaced with PB (100 µl) in 50% of assays, which were then incubated for 30 min. on the rotator. The assay then followed Protocol 1 (5.2.4.3).

5.2.4.4.2 Effects of increasing PAb concentration in assays

Assays were conducted to determine the optimal PAb concentration. In each 1 ml assay, stock PAb (1 µg/ml) was added at 50, 100, 200, 300, 400 & 500 ng/ml. The corresponding volume of buffer A was reduced in order to keep the 1 ml total assay volume, therefore the maximum PAb per assay was 500 ng/ml (and lacked any buffer A). The volume of magnetic beads (2×10^7 /ml) and blank sample (PBS) was maintained at 100 µl and 400 µl respectively. The assays were otherwise conducted as Protocol 1.

5.2.4.5 Protocol 1: a modification

As results from 5.2.4.4.2 showed an increased response with increased PAb, Protocol 1 was amended. The amount of PAb added to the 1 ml assay was increased from 100 to 300 ng/ml. Correspondingly, the amount of buffer A was reduced to 200 µl in order to maintain the 1 ml assay volume (which also contained 400 µl 'sample' and 100 µl of Ag-coated beads).

Additionally, it was found that aspirating supernatant solution by manual pipette could sometimes remove a small proportion of beads, which may have influenced results. Removal of solutions was better accomplished by sharply inverting the magnetic rack and blotting the inverted tubes on paper towels. This also ensured all reagents were promptly removed at the same time. This method was consequently employed for all further tests.

5.2.4.6 Detection of MTBE & assay repeatability

5.2.4.6.1 Detection of MTBE

Serial dilutions (1:10) of MTBE were made in PBS from 5000-0.5 ppm, PBS (lacking MTBE) was used as a blank control. Assays were performed with the increased PAb (300 ng/assay, with equal 30 min PAb/2Ab-HRP incubations in two separate experiments. Assays were conducted following Protocol 1, incorporating the modifications from 5.2.4.5.

At this stage, it was still unclear if low-end detection of MTBE (<50 ppm) was achievable and so further serial dilutions (1:10 in PBS) were made from the 50 ppm MTBE solution. A concentration range of 10-50 ppm was tested with PBS as a blank (zero MTBE). Assays were conducted following Protocol 1, with the modifications from 5.2.4.5.

5.2.4.6.2 Repeatability of the magnetic T-bead assay

The repeatability of the assays was then examined by conducting repeat tests (x 6) with 3 concentrations of MTBE (0, 0.5 & 5000 ppm in PBS). In addition to evaluating repeatability of the assay, the stability of the Ag-coated T-beads was also investigated. T-beads that had been stored at 4 °C for 7 days after Ag-coating were tested against a new batch of freshly Ag-coated beads. Serial dilutions of MTBE were made in PBS (0, 0.5 & 5000 ppm) and tested following Protocol 1, with modifications from 5.2.4.5.

5.2.4.7 Ag-coating T-beads: coating protocol revision

The bead-coating protocol was revised in order to economise on the use of both beads & Ag and supply 12 separate assays. The revised coating solution (1.3 ml) thus contained: 13 µl Ag (106 µg/ml), 130 µl magnetic beads (2×10^9 stock) and 1.157 ml buffer A. This cocktail gave a coating ratio of 1 µg Ag: 2×10^8 beads, in comparison to 1 µg Ag: 0.3×10^8 beads for the previous coating solution, which used 48 µg Ag to 1.48×10^9 beads. The cocktail was mixed by inverting the tubes (x 3), which were then incubated for 48 hr at RT on slow rotation (8 rpm). The beads were then washed and blocked as described in 5.2.4.2, they were resuspended in 1.3 ml buffer A, stored at 4 °C and diluted 1:10 in buffer A prior to use.

5.2.4.8 Effect of reducing Ag-coating concentration

To examine the effect of the Ag-coating concentration, three sets of beads were coated with Ag at 0.1, 0.5 & 1 µg per 2×10^8 beads. The coating procedure was otherwise as described in 5.2.4.7. Each set of beads were then tested separately and in duplicate with 3 MTBE concentrations (0, 50, 5000 ppm in PBS) with 2×10^7 beads in a total assay volume of 1 ml.

Assays were conducted following Protocol 1, incorporating modifications from 5.2.4.5 (concerning PAb concentration) up to the colour development stage, which was modified as follows:

A reduced volume of TMB (250 μ l instead of 500 μ l) was added to each tube, which was then capped and inverted (x 3) to mix. In contrast to Protocol 1, the tubes were then placed onto the rotator and mixed for 10 min. The Stop solution (also reduced to 250 μ l) was added to each tube consecutively. Tubes were then inverted to mix (x 3) and placed onto the magnetic rack for 1 min to remove beads from the reactant solution. As a result of the reduced final assay volume (500 μ l), only one 300 μ l aliquot was transferred to a microtitre plate well for reading of A450.

5.2.4.9 Protocol 1: a further modification

The reduced volume (500 μ l) of reactant, together with continuous mixing during the colour development stage gave improved assay signals. This modification was consequently retained for future assays. Results from 5.2.4.8 confirmed that 1 μ g: 2×10^8 beads was the optimum Ag coating concentration and this was also retained for future bead preparations.

5.2.4.10 Effect of increased PAb and incubation times

Two 6 assay tests were performed as Protocol 1, with 3 concentrations of MTBE (0, 50 & 5000 ppm in PBS, 5.1.1.1). As a result of previous findings, a second test was conducted incorporating the PAb at either 100 or 300 ng per 1 ml assay. PAb was incubated for 60 min. Assays were then continued as Protocol 1 (5.2.4.3).

5.2.4.11 Magnetic bead assay - Protocol 2

Protocol 2 incorporated the modifications to Protocol 1 obtained from the early development work. T-beads were coated as described in 5.2.4.2, although the revised coating procedure was used as described in 5.2.4.7. The full Assay Protocol 2 is described as follows:

All reagents were brought to RT, stock solutions of PAb & 2Ab-HRP (1 μ g/ml) were freshly prepared in buffer A prior to use. In a 2.5 ml Eppendorf, 400 μ l of sample, 200 μ l buffer A, 300 μ l PAb (300 ng) and 100 μ l of Ag-coated beads (1.48×10^7) were added and mixed by inversion (x 3). The tubes were then placed on the rotator and incubated for 1 hr at RT. Wash steps were then performed as 5.2.4.3.1.

A 1 ml volume of stock 2Ab-HRP (100 ng/ml) was added to each tube, again mixed by inversion and incubated on the rotator for 30 min at RT. This was followed by another sequence of three wash steps (as 5.2.4.3.1).

TMB (250 µl) was added to each tube, which was then capped and inverted (x 3) to mix. In contrast to Protocol 1, the tubes were then placed onto the rotator and mixed for 10 min. Stop solution (250 µl) was then added to each tube consecutively. Tubes were then inverted to mix (x 3) and placed onto the magnetic rack for 1 min to remove beads. One 300 µl aliquot was transferred to a microtitre plate well and the absorbance read at 450 nm.

This Protocol 2 was then used for the following investigations.

5.2.4.12 Assay performance with beads coated with 2 concentrations of Ag (Protocol 2)

Assays were conducted using beads freshly coated with 2 concentrations of Ag (1 & 10 µg per 2 x 10⁸ beads). A range of MTBE concentrations (0, 50 & 5000 ppm in PBS) were tested with both batches following Protocol 2.

5.2.4.13 Protocol 2: a further modification

The effect of adding T20 (at 0.2 % v/v) to the wash buffer C was investigated. The modified buffer is referred to as buffer C/T and was retained in Protocol 2. Due to the effectiveness of the revised buffer C (containing 0.2% v/v T20), this condition was retained in Protocol 2.

5.2.4.14 Detection of MTBE & MODMO by magnetic bead assay (Protocol 2)

Stock solutions (5000 ppm) of both MTBE and MODMO were made in PBS and both were serially diluted (1:10) down to 0.05 ppb. These solutions were each tested with the magnetic bead assay, following Protocol 2 incorporating buffer C/T (5.2.4.13). In order to test the full range of concentrations, two magnetic racks were used simultaneously (enabling 12 assays, one per concentration).

Lower-end dilutions of MODMO were used to compare the magnetic bead assay directly with the ELISA (Protocol 3, developed in 5.2.1.6). Each MODMO concentration was tested simultaneously by the magnetic bead assay (Protocol 2 using C/T WB) and by the ELISA (Protocol 3, 5.2.1.6). This then conclude the magnetic bead assay development.

CHAPTER 6

RESULTS (MTBE Immunoassay development)

6.1 INTRODUCTION

This chapter describes the results obtained from the second part of the experimental work described within this thesis. These results relate to the development of a novel enzyme-linked immunosorbent assay (ELISA) for the detection of MTBE in soil and water samples. This chapter is divided into four main areas. The first is dedicated to the initial development of the ELISA (6.2). Section 6.3 reports the further development of the MTBE ELISA by way of new protein conjugates. Sections 6.4-6.5 report the transposition of the ELISA into two alternative immunoassay (IA) formats; an enzyme-linked *flow-through* assay (ELIFA); and a magnetic bead immunoassay (MBI). Each section ends with a discussion.

6.2 ELISA

6.2.1 Step-by-step optimisation of Protocol 1 for the MTBE ELISA

Protocol 1 (5.2.1.2) was optimised with respect to several variables, as indicated in each experiment below. These experiments are listed chronologically and as a result of each, the optimised parameter was maintained throughout the subsequent experiments, unless specified differently.

6.2.1.1 Preliminary ELISAs using BSA-MTBE and BSA-MODMO Ag conjugates

Preliminary assays were performed using two antigen (Ag) conjugates; BSA-MTBE and BSA-MODMO (7-Methoxy-3, 7-dimethyloctanal), the aim being to determine that the primary antibody (PAb) raised against the BSA-MODMO could recognise both the Ag conjugates supplied. Ag-coated plates were tested with a single MTBE solution (5000 ppm in PBS, 5.2.1.3.1) and without MTBE (PBS). Non-specific binding (NSB) was investigated using control wells lacking either Ag, biotinylated primary antibody (PAb) and/or 5% BSA (w/v) blocking buffer (BB).

After repeated assays, the BSA-MTBE Ag conjugate proved unreactive, shown by the lack of distinction between assays with or without MTBE (Fig. 6.1). However, a clear distinction between the test solutions with the BSA-MODMO Ag was apparent. Maximum assay signals (0 ppm MTBE) were clearly higher than the controls (without Ag or PAb). Non-specific binding of the streptavidin-HRP (s-HRP) label was <0.05 (A450), rising to 0.22 in unblocked wells. Future

work was therefore continued using the BSA-MODMO Ag conjugate only. BSA-MTBE Ag was abandoned.

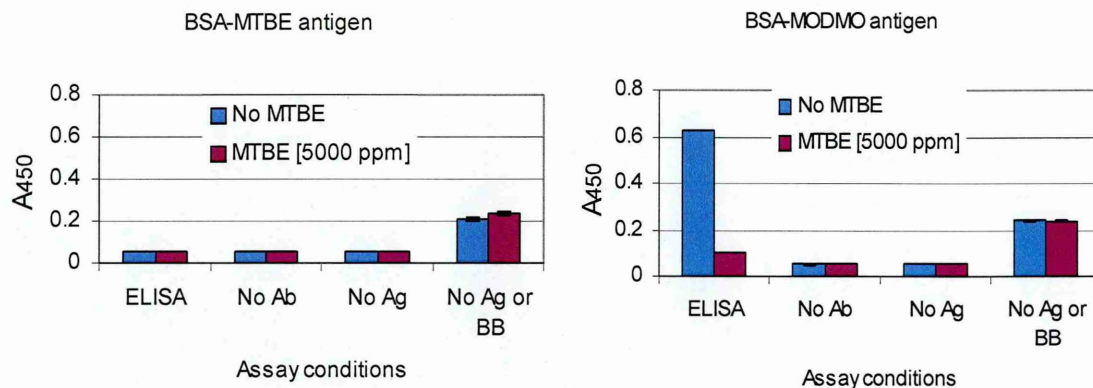


Fig. 6.1 Preliminary ELISAs using two BSA-Ag conjugates

BSA-MTBE Ag and BSA-MODMO Ag conjugate assays were compared, controls lacked Ag, biotinylated PAb and/or BB (5.2.1.3.1). The assay procedure was conducted as described in Protocol 1 (5.2.1.2). Results are presented as mean values $\pm$ SD, $n=8$.

6.2.1.2 PAb-Ag binding in different solutions

Assays were conducted to investigate the effects of four different solutions on the PAb-Ag binding step. These were: RO water, PBS, 5% BSA (w/v) solution and MTBE (5000 ppm in PBS, Fig. 6.2).

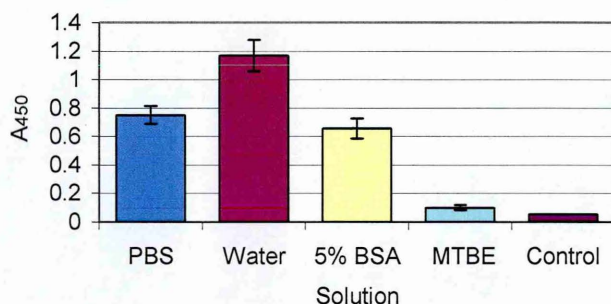


Fig. 6.2 PAb-Ag binding in different solutions

The assay procedure was conducted as described in Protocol 1 (5.2.1.2). Results are presented as mean values $\pm$ SD, $n=8$. Controls lacked Ag or PAb and were always <0.060 A450 (5.2.1.3.2). The CV was $<10\%$ in all substrates.

Highest signals were obtained when the PAb incubation was done in RO water (pH 6.7). However, the greatest inter-well variation also occurred under this condition. The SD of replicate experiments in RO water was 1.17, compared to 0.06 in PBS buffer. The relatively high response obtained with PAb in the 5% w/v BSA solution indicated that it preferentially bound to the immobilised Ag rather than to free BSA protein in solution.

6.2.1.3 Optimisation of concentration of BSA in blocking buffer (plate preparation)

Experiments were conducted to determine the optimal concentration of BSA in the blocking buffer (BB) during microtitre plate preparation (5.2.1.3.3). Plates were coated with Ag and blocked with 1% and 5% BSA (w/v) BB (Fig. 6.3).

Fig. 6.3 shows that the BSA blocking concentration could be reduced to 1% BSA (w/v) without before NSB increased. The dynamic range between zero and 5000 ppm MTBE improved, suggesting that a higher (>1%) BSA content might over-block the wells, perhaps smothering the Ag and so impeding the PAb recognition. PAb did not bind to free BSA *in solution* at either concentration (1% or 5% BSA solution), indicated by the high A₄₅₀ (Figs. 6.2-6.3). Future plates were coated with the BSA-MODMO Ag and blocked with a 1% BSA (w/v) BB solution.

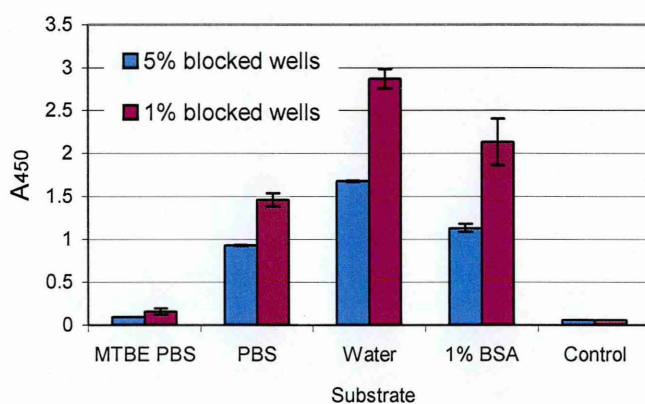


Fig. 6.3 Effect of reduced BSA concentration in blocking buffer

Two BSA-MODMO Ag coated plates were blocked using 1% and 5% BSA (w/v) BB. A range of PAb incubation substrates, including MTBE (5000 ppm in PBS) were tested in both plates (5.2.1.3.3). Assays were otherwise performed as in Protocol 1 (5.2.1.2). Controls lacked biotinylated PAb. Results are shown as mean values $\pm$ SD, $n=4$.

6.2.1.4 Optimisation of assay signal: colour development

Colour development based on the oxidation of TMB (5.1.1.1) by immobilised s-HRP was investigated. Assay signals were measured kinetically (A₆₃₀, 5.2.1.3.4) and after the addition of Stop solution (1 M H₂SO₄) at A₄₅₀. Higher signals (almost 3-fold) were obtained reading at A₄₅₀ ('stopped' reaction, Fig. 6.4).

Fig. 6.5 shows the rate of oxidation after PAb-Ag binding in different substrates. Colour development was quite linear over 20 min. for all the assays tested. The results (Fig. 6.5) showed that a more consistent assay response was achieved when PAb binding was carried out in PBS (10 mM) compared to other substrates. The consistency is shown in the reproducibility of the final rate of colour development.

Two alternative commercial colour development products (both based on TMB) were compared in Fig. 6.6. The TMB substrate solution (Sigma-Aldrich, Poole, Dorset, UK) that had been routinely used up to this point in the optimisation work was compared to another brand, 'TMBUS' (Moss Inc., 5.1.1.1), which contained a higher concentration of TMB - 2.08 M compared to 1.58 M reported for the Sigma product. A series of assays were conducted (Protocol 1) and the two TMB substrates were compared at the colour development stage. Comparison of the results obtained (Fig. 6.6) revealed a ~50% increase in absorbance (at 450 nm) with the TMBUS solution. The sensitivity of the assay was therefore substantially increased. The TMBUS solution (2.08 M) was consequently retained for use in all future ELISA work and allowed the colour development time to be reduced to only 10 min.

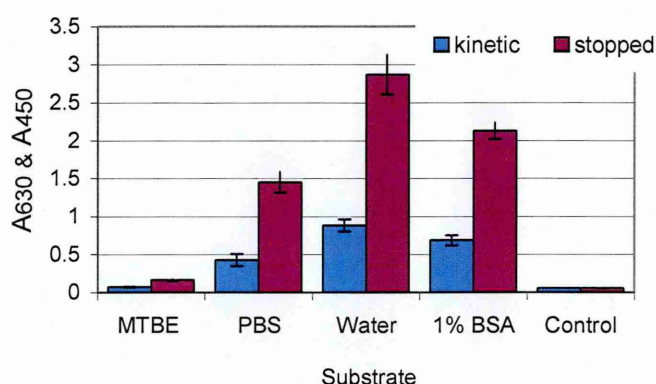


Fig. 6.4 Comparison of assay signal at two absorbency wavelengths

Assays were conducted as described in 5.2.1.3.4. The kinetic TMB reaction (A630) and the stopped TMB reaction (A450) were both assessed after 20 min. colour development. Controls lacked biotinylated PAb. Results are shown as mean values $\pm$ SD, $n=4$.

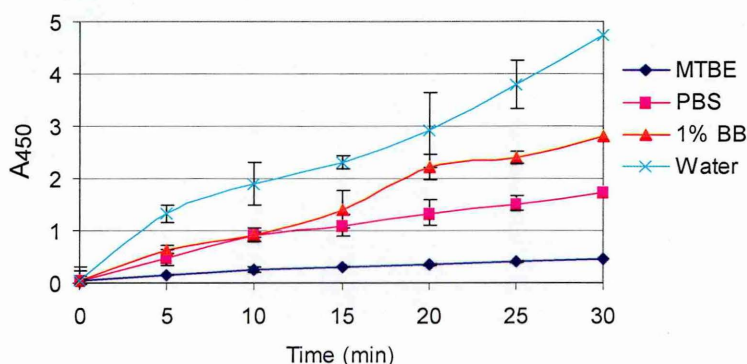


Fig. 6.5 Determination of optimal TMB incubation time

The assay procedure was conducted as described in Protocol 1 (5.2.1.2) up to the colour development step. A 100 μ l volume of TMB was added to the wells, then stopped with acid and read at 450 nm every 5 min (5.2.1.3.4). Assays including MTBE (5000 ppm) were used as controls. Results are shown as mean values $\pm$ SD, $n=8$.

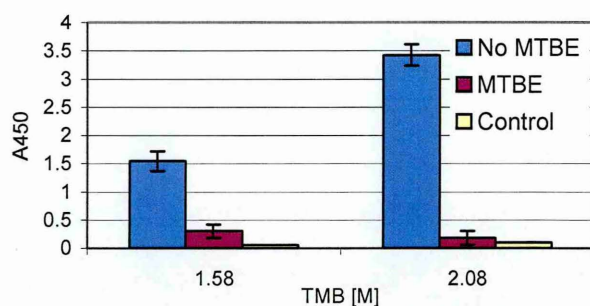


Fig. 6.6 Comparison of two TMB-based liquid substrate products

Two TMB liquid substrate solutions with different TMB molarities (1.58 and 2.08 M respectively) were compared (5.2.1.3.4). Colour development time was 15 min. The assay was otherwise performed as Protocol 1 (5.2.1.2). Controls were assays containing MTBE (5000 ppm) or assays lacking biotinylated PAb. Results are shown as mean values $\pm$ SD, $n=5$.

6.2.1.5 Effect of ionic strength on biotinylated PAb-Ag binding

As a more consistent assay response was achieved when PAb binding was carried out in PBS (10 mM), further experiments were conducted in which PAb-Ag binding was done in a range of PBS concentrations (5.2.1.3.5) to determine if assay reproducibility could be further improved (through changing the ionic strength of the buffer solution). A further series of full-plate assays (Fig. 6.8) were conducted in phosphate buffer (10 mM), but with reduced NaCl/KCl concentrations (no salt and 50% v/v salt).

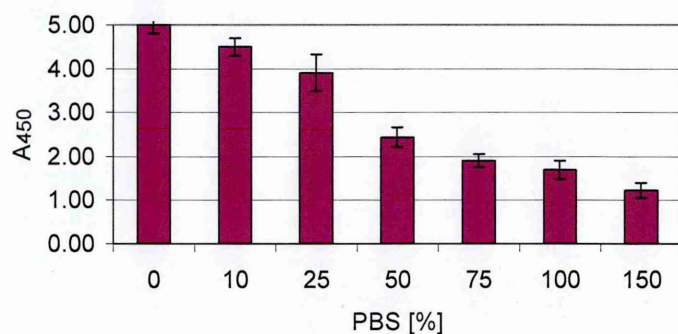


Fig. 6.7 Effect of ionic strength on biotinylated PAb-Ag binding

PBS buffer solutions of variable concentrations (%) were made by diluting with RO water (100% PBS is 10mM phosphate, 137 mM NaCl and 2.7 mM KCL, 5.2.1.3.5). The ELISA was conducted following Protocol 1 (5.2.1.2). Results are shown as mean values $\pm$ SD, $n=8$.

Fig. 6.7 shows that higher concentrations of PBS (high ionic strength) did indeed reduce assay responses, probably through interfering with protein-protein binding. However, Fig. 6.8 shows that greatest assay sensitivity to MTBE was found using standard PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl) for PAB-Ag incubation, with an IC₅₀ (50% B/Bo) of ~5 ppm MTBE, compared to ~45 ppm MTBE for PB (without any saline). The IC₅₀ is the dose or concentration (inhibitory concentration) of a compound to achieve 50% of maximal binding (A₄₅₀). This was

therefore retained as the preferred buffer for PAb, BSA-MODMO Ag, s-HRP and MTBE solutions.

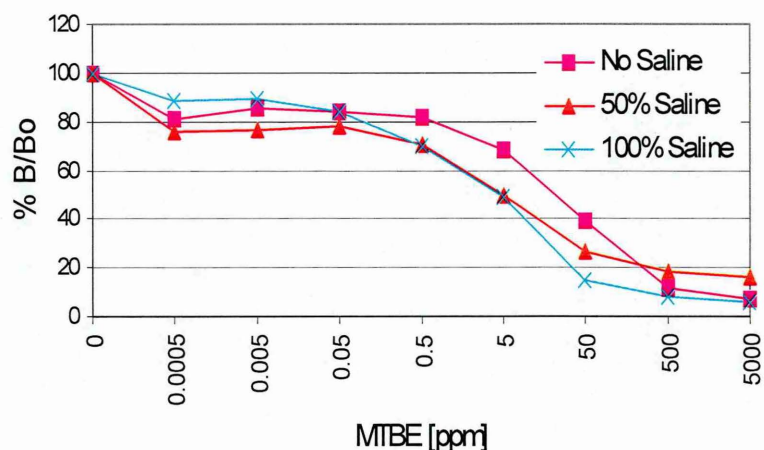


Fig. 6.8 Effect of saline on ELISA performance

Three full plate assays were performed with MTBE (0.0005-5000 ppm in either PB or PBS). Assay antibody incubations (PAb and s-HRP) were in phosphate buffer (10 mM) containing either 0, 50 or 100% of the usual saline content (100% is 137 mM NaCl and 2.7 mM KCl, 5.2.1.3.5). Assays were otherwise performed as Protocol 1, incorporating modifications up to 5.2.1.3.4 (Table 5.2). Results are presented as % B/Bo (5.2.1.1.2), $n=8$.

6.2.1.6 Effect of PAb and Ag concentrations on assay performance

An experiment was set up to determine the optimal PAb and Ag working concentrations to be used (Fig. 6.9). This took the form of a classical checkerboard assay, where Ag was coated at increasing concentrations across the wells (columns 1-12) and PAb was then diluted down (top-bottom (rows A-H) across these Ag concentrations. An extensive permutation of PAb-Ag concentration can thus be screened efficiently. A PAb concentration of 1000 ng/ml gave excellent responses and so this was retained as part of Protocol 1. Further ELISAs then were performed in the presence of MTBE (5.2.1.3.6) using two concentrations of Ag. These results are shown in Fig. 6.10.

Fig. 6.9 showed that an Ag concentration of 500 ng/ml gave very high assay responses with low inter-well variations using PAb concentrations of 250, 500 and 1000 ng/ml. This was further confirmed in Fig. 6.10, where assay responses were higher and more consistent using Ag at 500 ng/ml concentration (compared to 250 ng/ml). Concentrations of Ag at 500 ng/ml and PAb at 1000 ng/ml were therefore retained in order to achieve high assay response.

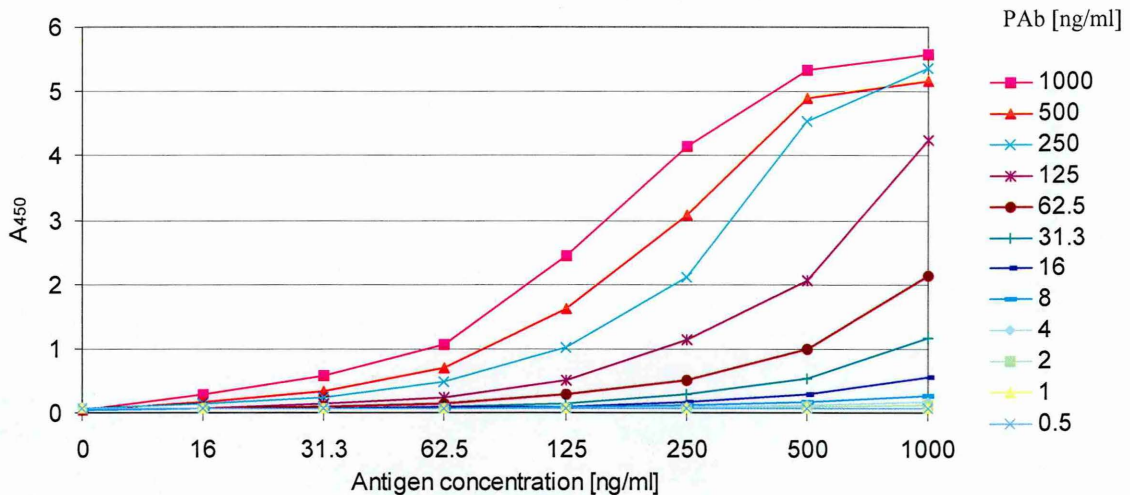


Fig. 6.9 Effect of PAb and Ag concentrations on assay performance

Serial Ag concentrations (0-1000 ng/ml) were tested with decreasing PAb concentrations (1000-0 ng/ml) diluted across Ag-coated wells (5.2.1.3.6). The assay procedure was otherwise conducted following Protocol 1 (5.2.1.2), incorporating modifications up to 5.2.1.3.5 (Table 5.2).

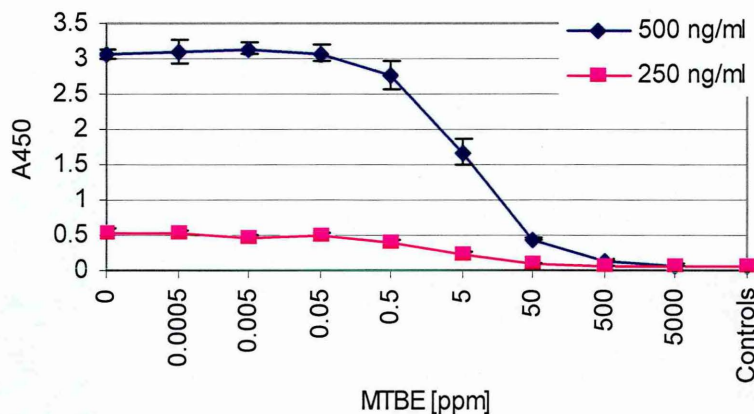


Fig. 6.10 ELISA responses with two Ag-coating concentrations and MTBE

Assays were performed using two Ag-coating concentrations (250 and 500 ng/ml, blocked with 1% BB, 5.2.1.3.6). The assays were otherwise conducted as Protocol 1 (5.2.1.2), incorporating modifications from Sections 5.2.1.3.1-5.2.1.3.5. Controls lacked PAb. Results are shown as mean values $\pm$ SD, $n=8$. The mean CV was 6.2% (500 ng/ml) and 6.8% (250 ng/ml) respectively.

6.2.1.7 Combined incubation of biotinylated PAb and s-HRP

Ag-coated plates were prepared and blocked using an increased volume (300 μ l) of BB. To try to reduce the overall assay time, both PAb and s-HRP were added to MTBE solutions and incubated together in the wells for 1 hr (5.2.1.3.7). By eliminating the secondary incubation step (with s-HRP), the overall assay time could be potentially reduced by 30 min. A standard ELISA was performed following Protocol 1, incorporating modifications up to 5.2.1.3.5 (Table 5.2).

Fig. 6.11 shows that the assay *could* distinguish between different concentrations of MTBE when both PAb and s-HRP were incubated together. However, the assay response fell considerably with a maximum signal (without MTBE) of 0.15 (A450), which was only 5% of the assay signal obtained from the standard ELISA using separate PAb and s-HRP incubation stages. It is possible that simultaneous PAb capture by the s-HRP may have induced a change in PAb conformation, which may have reduced its affinity for Ag binding. It is also possible that the presence of free s-HRP interferes in some way with PAb-Ag binding.

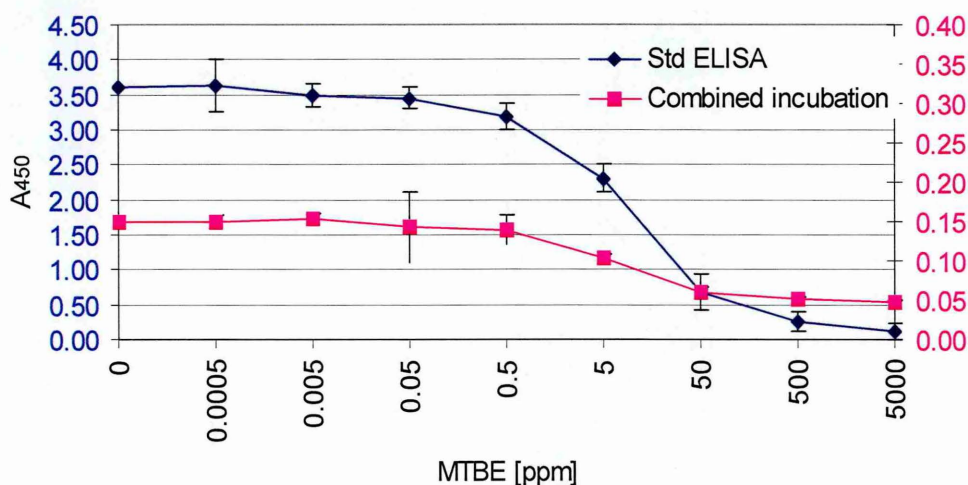


Fig. 6.11 Effect of combined incubation of biotinylated PAb and s-HRP

Ag-coated plates were blocked with 300 μ l BB and incubation of PAb and s-HRP were combined (5.2.1.3.7). A standard assay was also conducted (as the modified Protocol 1) in which incubation of the s-HRP was done after PAb incubation. The standard ELISA data sets should be read against the primary y-axis (left); the combined incubation data should be read against the secondary y-axis (right). Maximum absorbance for the combined incubation was only 0.15 (A450). Results are shown as mean values, $\pm$ SD, $n=8$. Mean CV values were 16.7% and 7.9% respectively.

6.2.1.8 Repeatability of the ELISA following the optimised Protocol

Two full-plate assays (5.2.1.3.8) were conducted following the optimised Protocol 1, incorporating modifications from 5.2.1.3.1-5.2.1.3.7) in order to determine the repeatability and accuracy of the MTBE ELISA. Fig. 6.12 shows that there was a 10-fold difference in sensitivity between the two plates; plate A shows an IC₅₀ (50% B/B₀) of only 50 ppm MTBE and the IC₅₀ of plate B was ~500 ppm MTBE. Whilst overall, the assay signals had been greatly improved, this variation of accuracy was unsatisfactory and so further assay development work was undertaken (6.2.2).

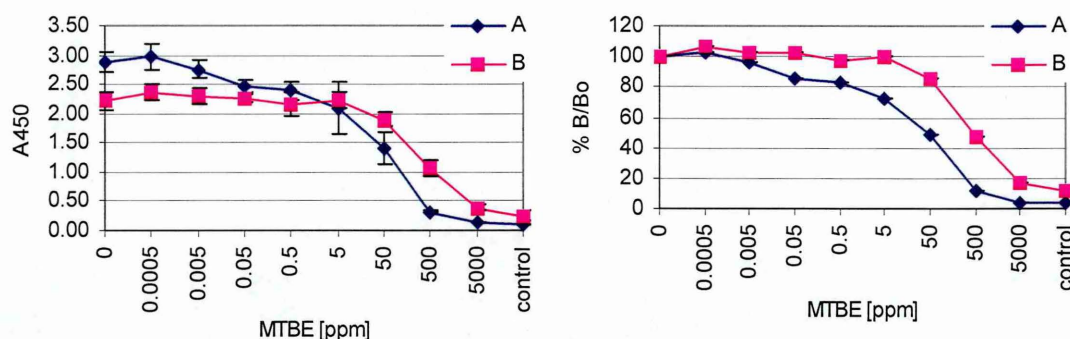


Fig. 6.12 Repeatability of the ELISA following the optimised Protocol 1

Two full plate assays (A and B) were conducted following the optimised Protocol 1 (incorporating modifications up to 5.2.1.3.7 (Table 5.2). A) Mean absorbance values from both plates are shown, $\pm$ SD, $n=8$; B) The %B/Bo calculated for both assays. The CV of each assay was $<10\%$. This work concluded the initial optimisation of Protocol 1. The optimised ELISA is hereafter referred to as Protocol 2 and the method is given in 5.2.1.4.

6.2.2 Protocol 2: Further optimisations

Protocol 3 was derived from Protocol 2 (5.2.1.4) by replacing the PAb with native (unconjugated) PAb. In order to detect and label this PAb (IgG mouse), a commercial, secondary antibody (polyclonal goat anti-IgG mouse) conjugated to HRP was used in place of the s-HRP. The experiments that resulted in these changes are described in the next four sections.

6.2.2.1 Comparison of two s-HRP conjugates at Ag-coating concentrations of 250 and 500 ng/ml

Following Protocol 2 (5.2.1.4), assays were performed using two batches of s-HRP conjugates (500 ng/ml) from two independent suppliers, Dako and Prozyme (5.1.1.2). Full plate assays were conducted on plates coated with two Ag concentrations (250 and 500 ng/ml, 1% BB) as selected in 6.2.1.6.

There were striking differences between the two commercial s-HRP products (Fig. 6.13), which was probably a reflection of the different conjugation procedures used by the different manufacturers. Although both were diluted to the same concentration (500 ng/ml), there was a $\sim 1:4$ difference in assay signal. Whilst the Dako (5.1.1.2) s-HRP had been used to this point, it was desirable to increase the assay signal (A450) and the Prozyme s-HRP clearly achieved this. However, there were significant variations between wells using the more active Prozyme s-HRP. Repeated assays were made but it was not possible to reduce these variations, even with assays (full plate) conducted using lower concentrations of the Prozyme reagent (not shown). Consequently, the CV increased to unacceptable levels ($\geq 50\%$) and repeatable sigmoidal curves

were not achieved. The instability of the Prozyme s-HRP could well have been the cause of the variation between the two full plate assays shown in Fig. 6.12. In light of these findings, an alternative method of PAb detection and HRP-labelling was investigated (6.2.2.2).

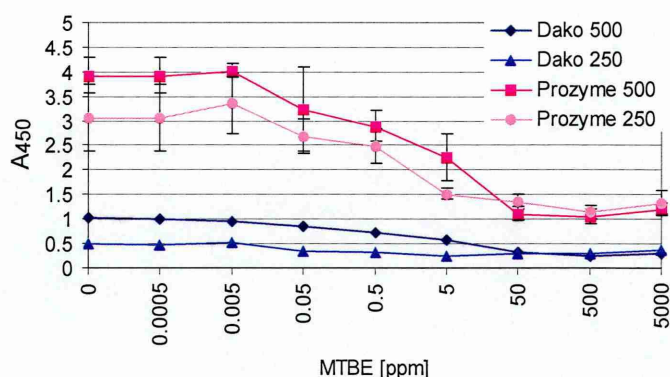


Fig. 6.13 Comparison of two s-HRP conjugates

ELISA plates were coated with two Ag concentrations (250 and 500 ng/ml) and blocked with 1% BB. Full plate assays were conducted following Protocol 2 (5.2.1.4). Two types of s-HRP were compared (5.2.1.5.1). Results are shown as mean values $\pm$ SD, $n=8$.

6.2.2.2 Detection of native (non-biotinylated) PAb using secondary HRP-labelled antibody (2Ab-HRP)

An alternative method of PAb detection and labelling was investigated by replacing the PAb with native (non-biotinylated) PAb (monoclonal IgG mouse). In order to label any captured PAb, a polyclonal secondary antibody (goat, anti-IgG mouse) - HRP conjugate was used (5.2.1.5.2). Fig. 6.14 shows a comparison of these two methods. Optimisation of both PAb and Ag concentrations were examined in 6.2.2.2.1.

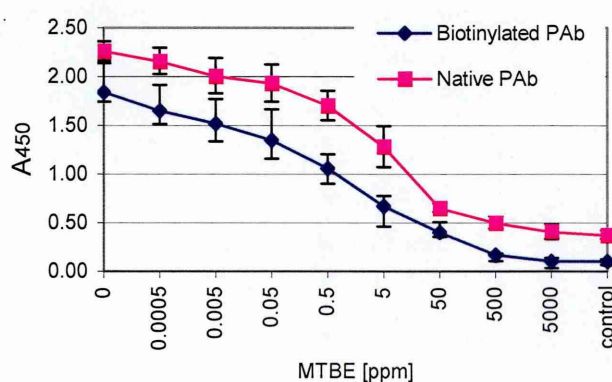


Fig. 6.14 Detection of native (non-biotinylated) PAb using 2Ab-HRP (anti-IgG mouse)

A standard assay (Protocol 2) was conducted. In the second assay, native (unconjugated) PAb and 2Ab-HRP (goat polyclonal IgG to mouse IgG) were used, diluted to 500 ng/ml (5.2.1.5.2). A range of MTBE solutions (0-5,000 ppm) were tested. Controls lacked PAb. Results are shown as mean values $\pm$ SD, $n=8$. The mean CV was 18.4% (biotinylated PAb) and 10.3% (native PAb).

6.2.2.2.1 Determination of native PAb and Ag concentrations

Assays were conducted to determine the optimal native PAb concentration for use with Ag-coated plates (5.2.1.5.3). Two Ag coating concentrations were tested (Fig. 6.15).

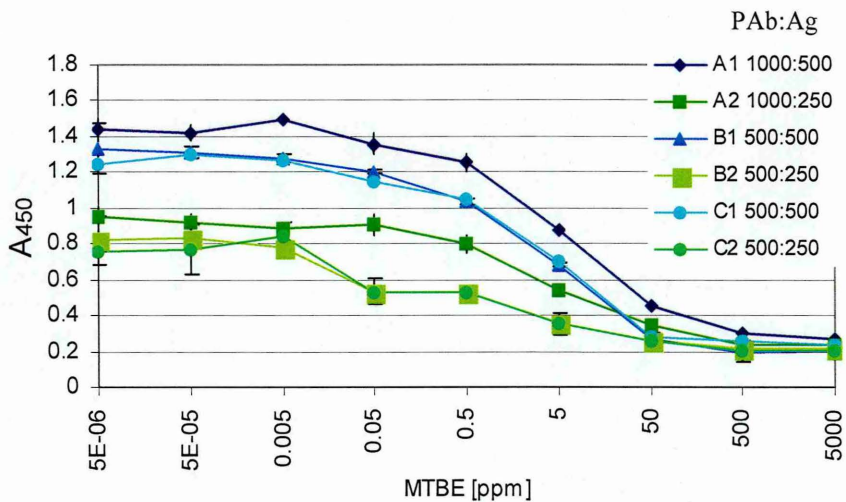
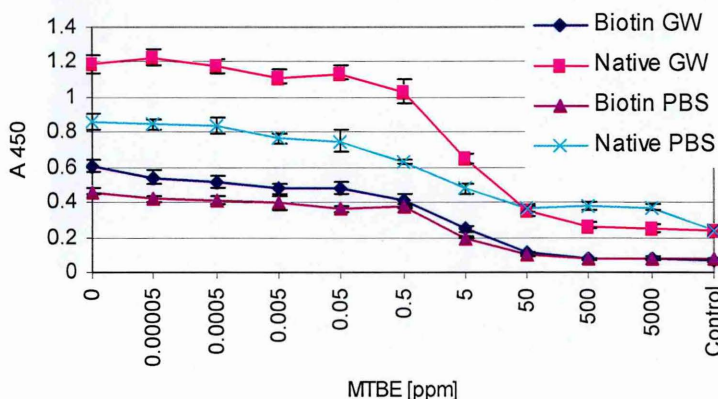


Fig. 6.15 Determination of native PAb and Ag concentrations

Native PAb (500 and 1000 ng/ml) was tested with two Ag concentrations. The legend key shows the ratio of PAb:Ag (ng/ml). Assays C1 and C2 were repeats of B1 and B2. Results are shown as mean values, $\pm$ SD, $n=8$. The CV was $< 9\%$ for all assays.

Fig. 6.15 shows that the highest and most reproducible assay responses were obtained using Ag at 500 ng/ml and native PAb at 500 ng/ml. For further confirmation of the improved assay performance using native PAb and the 2Ab-HRP, assays were conducted with reduced Ag-coating concentrations (250 ng/ml). The concentration of native PAb (500 ng/ml) was half that of the biotinylated PAb (5.2.1.5.4).



PAb type & substrate	Mean $\pm$ SD	Mean CV(%)
Biotin GW	0.02	6.25
Native GW	0.03	5.99
Biotin PBS	0.01	5.60
Native PBS	0.03	5.16

Fig. 6.16 Comparison of native and biotinylated PAb at reduced Ag-coating (250 ng/ml)

Results are shown of assays based on either native PAb (500 ng/ml) or biotinylated (1000 ng/ml) PAb with 250 ng/ml Ag. PAb labelling was by 2Ab-HRP or s-HRP respectively (500 ng/ml). MTBE solutions were made in either PBS or local groundwater (GW). The assay was otherwise run as Protocol 2. Controls lacked Ag. Mean values $\pm$ SD are shown and the CVs are shown on the right, $n=8$.

From these results, it can be seen that using native PAb improved assay response, yet used 50% less PAb per test. Additionally, the chemistry of the biotinylation (to the PAb) could be avoided. Therefore, these conditions were retained for future work.

6.2.2.2.2 Non-specific binding of PAb and 2Ab-HRP

NSB of the native PAb and 2Ab-HRP (both at 500 ng/ml) was investigated by comparing an assay (without MTBE) with a number of controls (5.2.1.5.5).

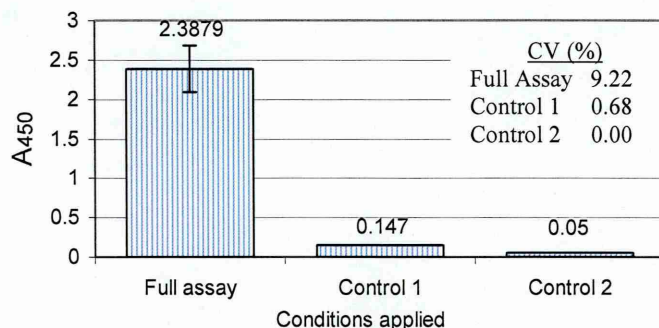


Fig. 6.17 NSB of native PAb and 2Ab-HRP

Plates were coated (500 ng/ml Ag) as Protocol 2 (5.2.1.4). An ELISA with no MTBE was conducted with two controls (5.2.1.5.5); Control 1 lacked PAb; Control 2 lacked 2Ab-HRP. Equivalent volumes (100 μ l) of PBS were used in place of each reagent where required. The assay was otherwise conducted as Protocol 2. Results are shown as mean values $\pm$ SD, n=4. The CV is indicated in the legend.

6.2.2.2.3 Combined incubation of native PAb and 2Ab-HRP

As with the biotinylated PAb (6.2.1.7), in an attempt to reduce assay time, combined incubations of both the native PAb and 2Ab-HRP were investigated (Fig. 6.18).

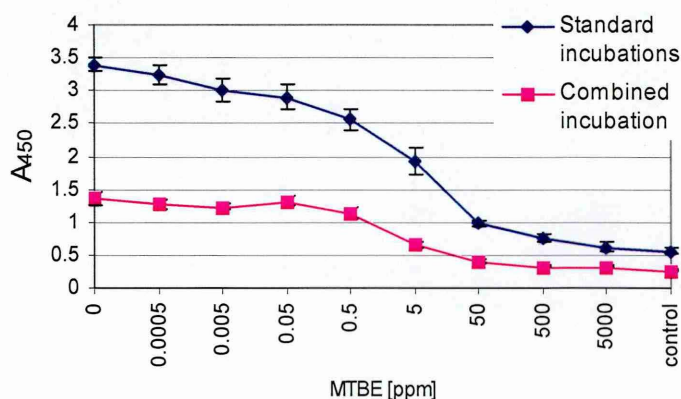


Fig. 6.18 Effect of combined incubation of native PAb and 2Ab-HRP

Ag-coated plates were blocked with 1% BB (300 μ l). Native PAb and 2Ab-HRP were combined and incubated (in the wells) for 1 hr (5.2.1.5.6). A standard full plate assay was also conducted (Protocol 2). Maximum absorbance for the combined antibody incubation was 1.36 (A₄₅₀). Results are shown as mean values, $\pm$ SD, n=8. Mean CV values were <6.9% for both assays.

Combined incubations resulted in a 60% decrease in signal (without MTBE). However, assay sensitivity to MTBE was unaffected. This was not such a drastic decrease as was obtained from co-incubation of biotinylated PAb and s-HRP, after which 95% of signal was lost (6.2.1.7). It was therefore concluded that the sequential method (two separate incubation steps) was necessary.

6.2.2.2.4 Reduction of native PAb incubation time

It is generally accepted that the PAb-Ag binding process occurs within 30 min in standard microtitre well assay formats (Butler & Engelmann, 2002). The incubation time of native PAb with Ag was consequently reduced from 1 hr to 35 min.

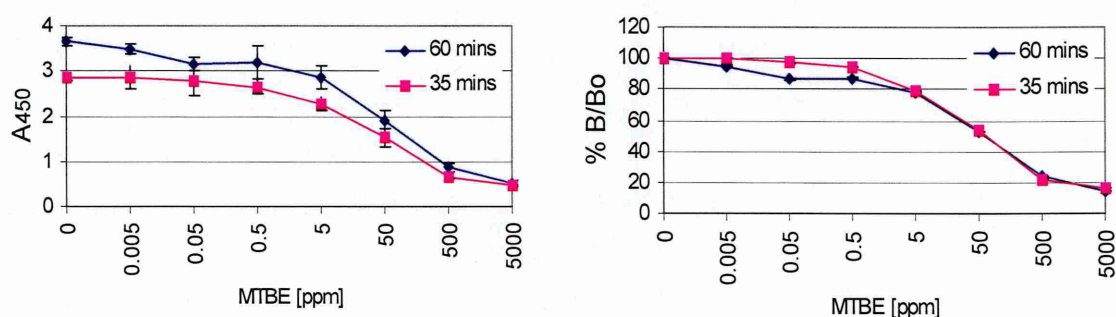


Fig. 6.19 Effect of reduced PAb incubation time

Two full plate assays were conducted with PAb (and MTBE) incubations of 35 and 60 min. A new ‘Ultrasensitive’ TMB solution was used (5.1.1.1), with colour development reduced from 10 min to 3 min (5.2.1.5.7). Assays were otherwise conducted as Protocol 2. Results are shown as mean values $\pm$ SD, $n=8$. The CV was $<8\%$.

Although some loss of signal was seen with a reduced PAb incubation time of 35 min, there was less inter-well variation, producing a smoother sigmoidal curve, indicating that the assay time could be reduced without compromising assay sensitivity. A450 readings were still close to the maximum capability of the plate reader (3.0).

This work concluded the further optimisation of Protocol 2. The optimised MTBE ELISA protocol gave an assay sensitivity range of ~ 0.5 -5000 ppm. The optimised ELISA is hereafter referred to as Protocol 3 and is detailed in 5.2.1.6.

6.2.3 ELISA Protocol 3: Stability of reagents and repeatability

To determine the repeatability and accuracy of the optimised MTBE ELISA using native PAb and 2Ab-HRP, three independent assays were performed using Protocol 3 (5.2.1.6) with the full range of spiked MTBE concentrations (Fig. 6.20). Further assays investigated the effects of temperature (Fig. 6.21), buffer (Fig. 6.22) and pH on assay performance (Fig. 6.23).

The optimised assay showed a linear range of 0.5-50 ppm, with increased sensitivity (IC₅₀ of 5 ppm MTBE), $R = 0.992$ (Fig. 6. 20). Higher temperatures were considered inappropriate for a field test method, as this would require some form of temperature control. Similarly, low temperatures significantly slow the PAb-Ag binding rates and enzyme activity. Therefore, an ambient RT (22 °C) was considered appropriate.

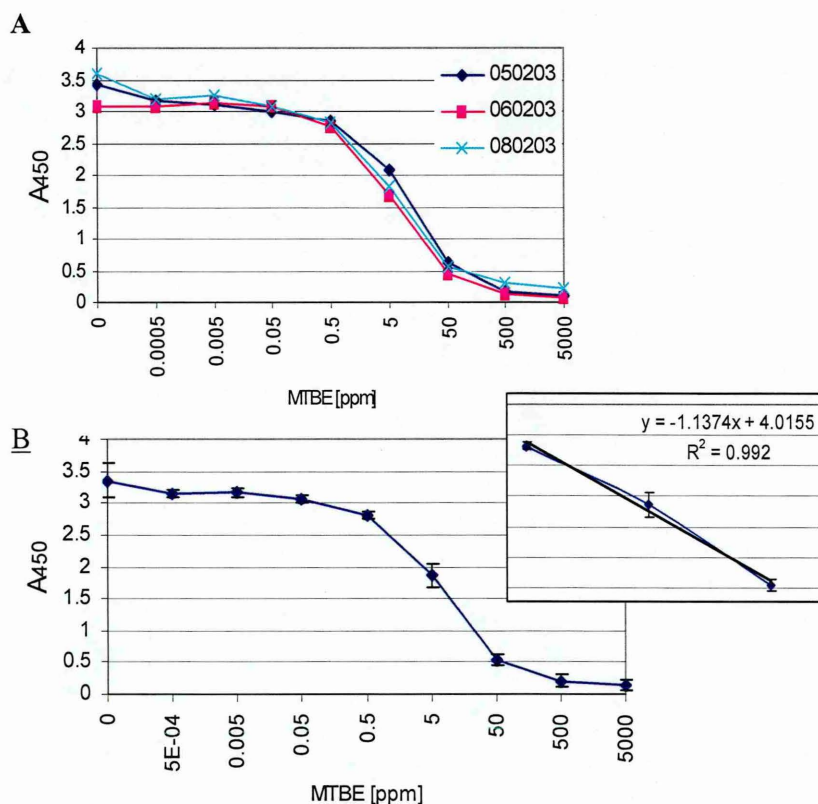


Fig. 6.20 Repeatability of three ELISAs with Protocol 3

(A) The results of three full-plate assays, each point is shown as a mean value $\pm$ SD, $n=8$.

(B) Mean value of all three assays, each point represents 24 absorbance readings. The best fit line between 0.5-50 ppm MTBE concentrations, the linear regression equation and R^2 values are shown in the right-hand inset (units and scale as per main graph). The CV was $\leq 10\%$.

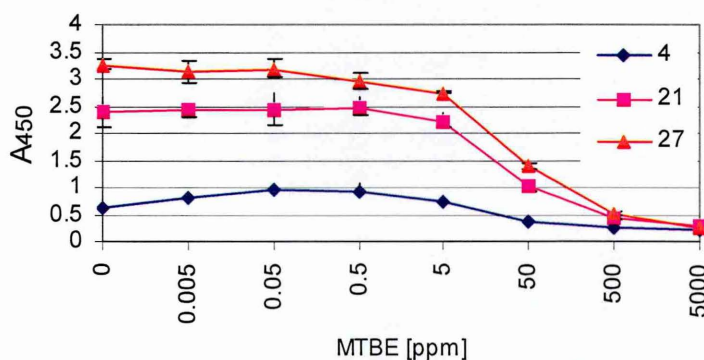


Fig. 6.21 Assay response at different temperatures

Three assays were conducted at different temperatures (4, 21 and 27 °C). These temperatures were selected to reflect approximate seasonal UK variation (5.2.1.6.2). Results are shown as mean values, $\pm$ SD, $n=8$; CVs were 8.29, 8.69 and 5.51% respectively.

Fig. 6.22 shows that greater inter-well variations occurred in saline-free buffers (PB and Tris). This confirmed the previous finding that a small proportion of salt actually minimised inter-well assay variation (Fig. 6.10), although some loss of signal occurred. However, as the optimised Protocol 3 involving PBS gave a response ≥ 3 (A450), which was the upper threshold of the plate reader, PBS was retained as the preferred buffer for reagents and MTBE solutions.

Fig. 6.23 shows that results were more variable at the higher pH 9. This was most likely due to instability of the reagents as many proteins destabilise and denature at this pH. Interference from organic matter within the unfiltered groundwater samples may also have increased the optical density.

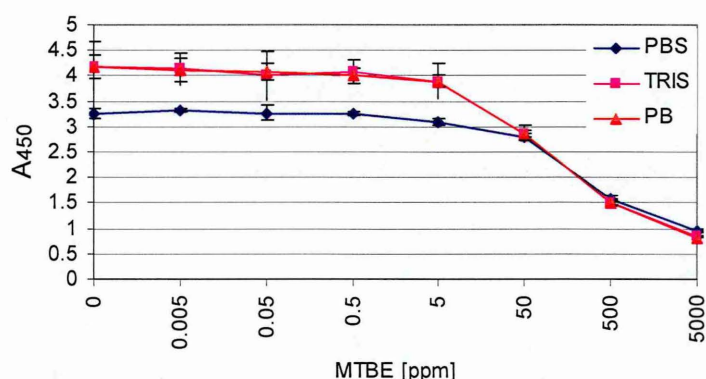


Fig. 6.22 Assay response with 3 alternative buffers

Antibodies and MTBE concentrations were prepared in PBS, Tris or PB (all were 10 mM, pH 7.4, 5.2.1.6.2). Results are shown as mean values $\pm$ SD, $n=8$. CVs were: PBS 2.69, Tris 8.32 and PB 7.83 %.

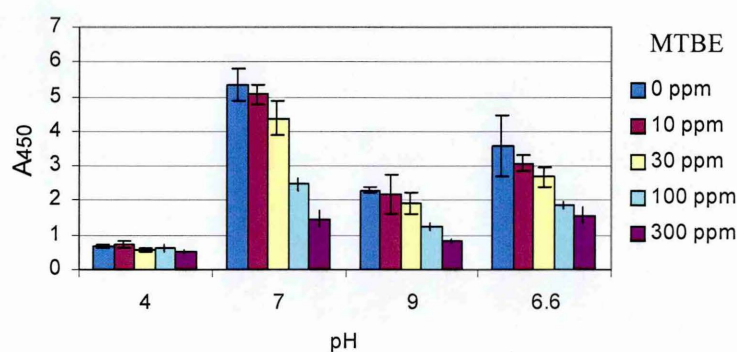


Fig. 6.23 Effect of pH on assay response

Three buffer solutions were prepared from buffer standards (5.2.1.6.2) at pH 4, 7 and 9. MTBE concentrations were also prepared in an unbuffered local groundwater (pH 6.6) and was tested with both antibodies diluted in PBS. MTBE concentrations (ppm) are shown in the legend key. Results are shown as mean values $\pm$ SD, $n=8$.

This concluded the developmental work for the MTBE ELISA. The following results relate to further characterisation of the ELISA format in relation to (i) the stability of MTBE solutions, (ii) cross-reactivity with related and/or structurally similar organic compounds and (iii) detection of MTBE in real environmental samples.

6.2.4 Stability of MTBE in buffer (spiked PBS)

Due to reports of MTBE evaporating from water, a series of experiments were conducted to investigate the rate at which MTBE was lost from solutions. This has implications for potential under-reporting of MTBE concentrations using the ELISA method. Nitrogen gas was bubbled through a MTBE solution (10 ml, 5000 ppm) and the rate and quantity of MTBE loss was examined (5.2.1.7.1). Fig. 6.24 shows a linear loss of MTBE, with time, through forced evaporation. MTBE content could still be determined from the standard calibration curve after 120 min of N₂ bubbling, after which the level of MTBE was undetectable, being below the minimum assay range of 0.5 ppm.

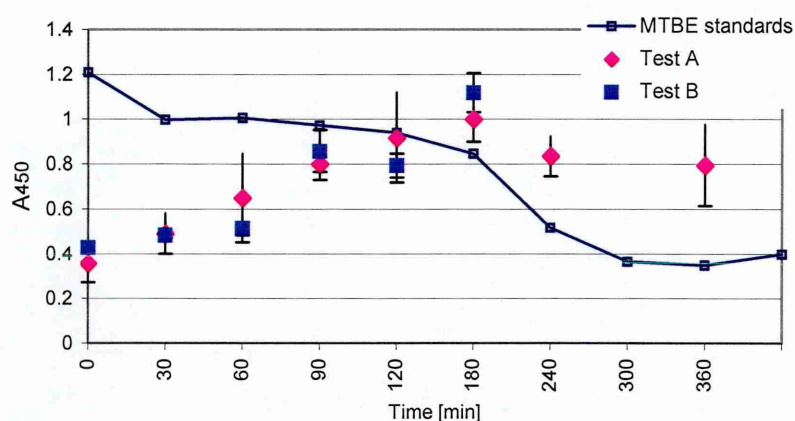


Fig. 6.24 MTBE losses from PBS buffer through volatilisation

Nitrogen gas was bubbled through a MTBE solution (5000 ppm) from which samples were withdrawn at 30 min intervals and tested by ELISA (5.2.1.7.1). Two sets of results (A and B) are shown (mean values are plotted $\pm$ SD, $n=4$). A standard full plate MTBE ELISA was then run using fresh MTBE solutions and the results have been superimposed on the graph to show the calibration curve from which sample concentrations were determined (mean values, $n=8$).

6.2.4.1 Stability of MTBE solutions over time

A range of MTBE solutions were prepared in PBS and stored at RT (22 °C) over a 3-week period (5.2.1.7.2). These were tested periodically against freshly prepared solutions (Fig. 6.25).

Fig. 6.25 shows that assay response was more consistent when the MTBE solution had been aged for 3 hours after preparation, suggesting that the compound dissolution is not instantaneous and requires longer for a truly homogeneous solution (this is supported by

observation). Once in solution, concentrations remained stable in PBS (at RT) over the three-week period.

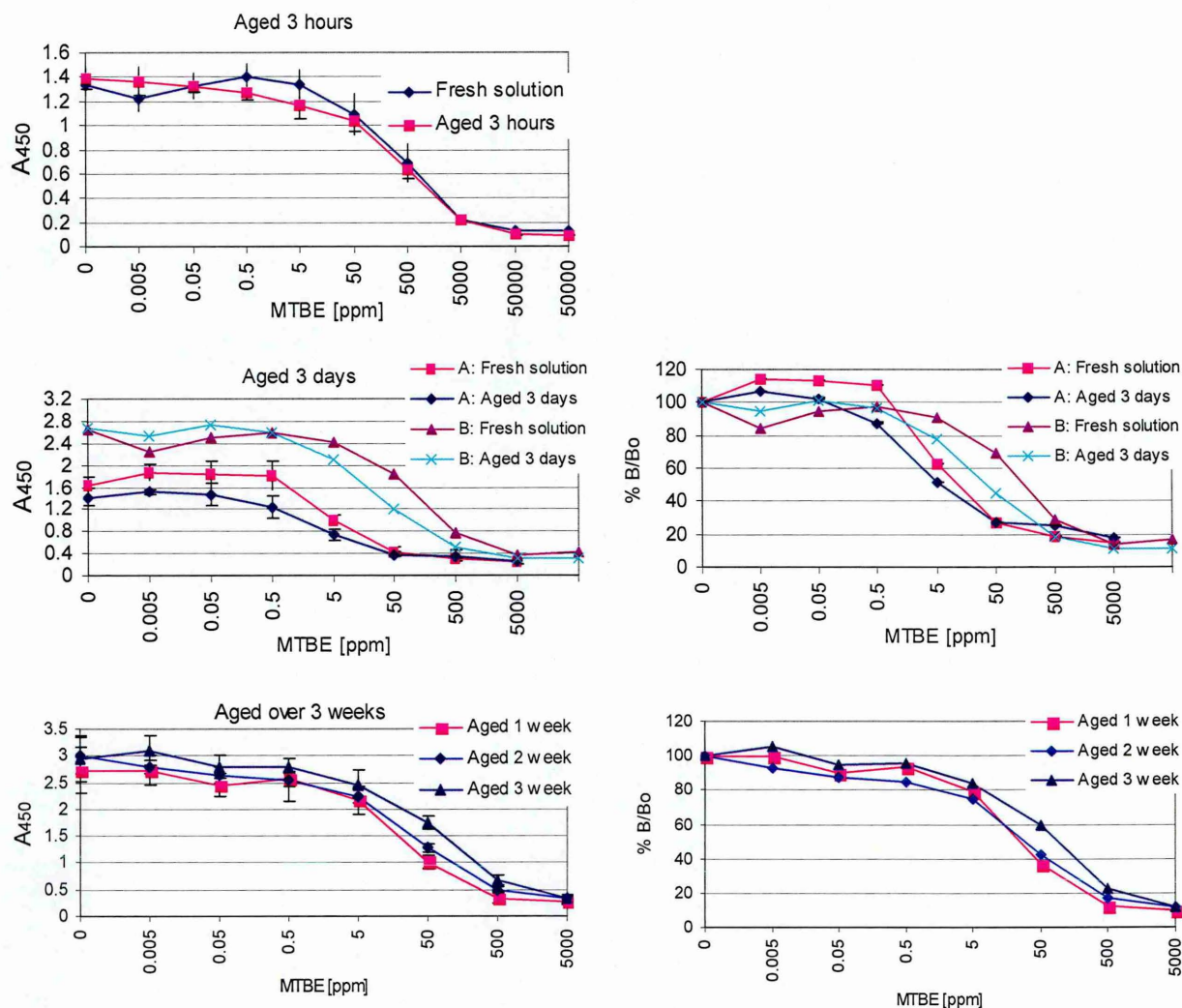


Fig. 6.25 Stability of MTBE solutions over time

MTBE solutions (0-5000 ppm) were prepared in PBS (5.2.1.7.2) and stored at RT (22 °C). The aged solutions were then periodically assayed and compared with fresh MTBE solutions (see individual chart titles). Full plate assays were conducted on each occasion, results are shown as mean values $\pm$ SD, $n=8$. The CV was $<10\%$ for each assay.

6.2.5 Detection of fuels and alternative oxygenates

The following experiments (5.2.1.8) investigated the specificity of the MTBE ELISA. Unleaded petrol and diesel fuels were tested, as well as several oxygenate alternatives; ethanol (EtOH) and methanol (MeOH), *tert* amyl methyl ether (TAME), *di*-isopropyl ether (DIPE) & ethyl *tert* butyl ether (ETBE). MTBE and its breakdown products; *tert* butyl ether (TBA) and *tert* butyl formate (TBF) were also tested. Protocol 3 (5.2.1.6) was followed for all experiments.

No cross-reactivity was found with either MeOH or EtOH. In Fig. 6.26, a response to unleaded petrol occurred with petrol mixtures of 500 and 5000 ppm. Repeat experiments found MTBE could be detected in petrol mixtures up to 10,000 ppm. MTBE content in fuels can be up to 15%, however, in the UK, this figure is generally between 5-10% (Squillace *et al.*, 1995). Higher petrol concentrations produced unreliable results, probably due to the presence of an excess of organic-phase hydrocarbon and its detrimental effect on PAb binding reaction. Diesel (with zero oxygenate content) did not elicit a response.

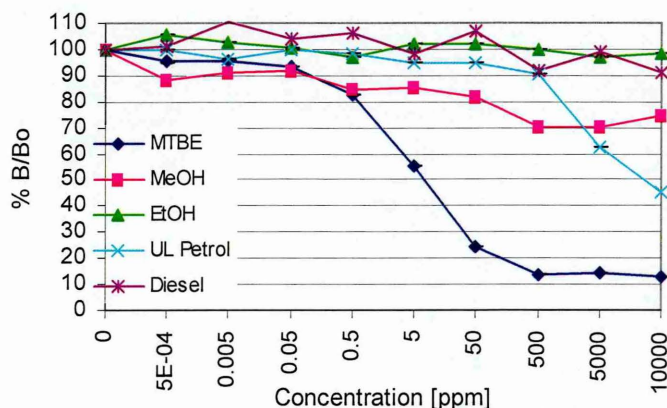


Fig. 6.26 ELISA response to fuels and oxygenate alternatives

Unleaded petrol, diesel, methanol, ethanol and MTBE mixtures were prepared in PBS buffer (0-5000 ppm) and tested by ELISA (5.2.1.8). Results are shown as mean values $\pm$ SD, $n=8$. Repeated assays with unleaded petrol mixtures (at 10,000 ppm) produced a response of 45% B/Bo, indicated that the dynamic range was between 500-10,000 ppm.

In contrast, Fig. 6.27 shows that the ELISA can also detect several fuel oxygenate alternatives; TAME, DIPE and ETBE. MTBE breakdown products TBA and TBF can also be detected (Fig. 6.28). However, in all cases, the PAb shows greatest affinity for free MTBE (as anticipated) and this is reflected in the fact that the assay is most sensitive to this compound. The IC₅₀s (%B/Bo) were, in order of sensitivity: MTBE 8 ppm, TBF 60 ppm and TBA ~240 ppm.

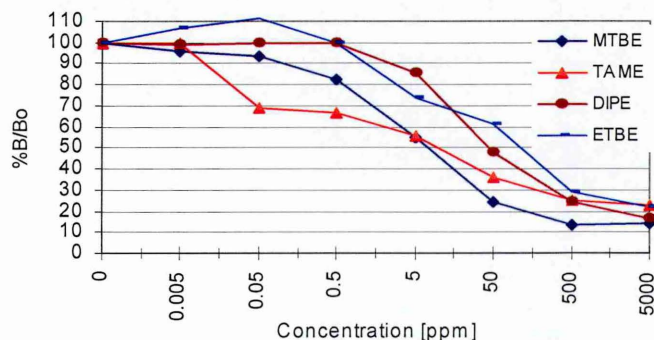


Fig. 6.27 ELISA response to alternative fuel oxygenates

MTBE, TAME, DIPE and ETBE solutions were prepared in PBS buffer (0-5000 ppm) and tested by ELISA (5.2.1.8) using Protocol 3 (5.2.1.6). Results are shown as mean values $\pm$ SD, $n=8$.

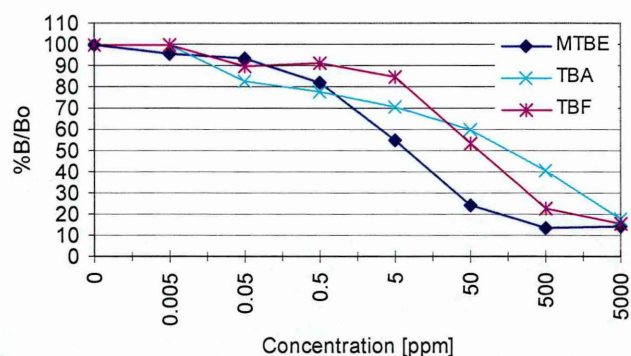


Fig. 6.28 ELISA response to MTBE and breakdown products

MTBE, TBA and TBF solutions were prepared in PBS buffer (0-5000 ppm) and tested by ELISA (5.2.1.8) using Protocol 3 (5.2.1.6). Results are shown as mean values $\pm$ SD, $n=8$.

6.2.6 Investigation of PAb binding characteristics with MODMO-structural analogues

Previous development work had concentrated primarily on MTBE detection. However, the PAb had been raised against a BSA-MODMO Ag conjugate (MODMO is 7-Methoxy-3, 7-dimethyloctanal, its structure is shown in Fig. 6.34). Assays were consequently conducted to determine the assay response to free MODMO (Fig. 6.29) in comparison to MTBE (5.2.1.9.1). Further assays looked at lower-end MODMO concentrations (Fig. 6.30).

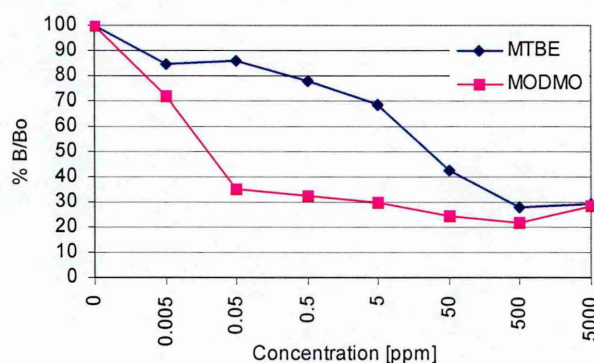


Fig. 6.29 Assay response to free MODMO and MTBE

MTBE and MODMO solutions were prepared in PBS (5.2.1.9.1). Two full plate assays were performed as Protocol 3 (5.2.1.6). Results are shown as mean values $\pm$ SD, $n=8$. The CV was 13% for both MTBE and MODMO determinations.

These results clearly show that the affinity of the PAb is for the analyte it was raised against, MODMO. Consequently, detection of this compound was possible down to ~ 5 ppb, a 1000 fold increase in sensitivity compared to MTBE detection.

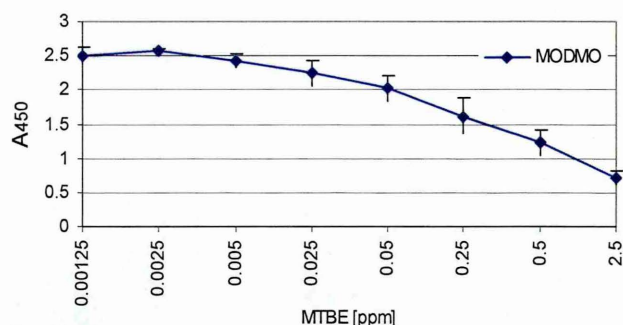


Fig. 6.30 Assay response to lower-end MODMO concentrations

Further dilutions were made from a single MODMO solution (5 ppm) used in Fig. 6.28 (5.2.1.9.1). Assays were performed as Protocol 3. Results are shown as mean values $\pm$ SD, $n=4$. The CV was $\leq 9\%$.

A subsequent expansion of this work led to further investigations (5.2.1.9.2) in order to gain some insight into how the PAb bound to MODMO, MTBE and the other cross-reacting fuel oxygenates (6.2.5) by testing structurally similar compounds. Two principle aspects of chemical structure were addressed:

1. The importance of the straight carbon chain length (Fig. 6.31)
2. The importance of the position of the $-OH$ group (1^{st} or 2^{nd} carbon) and of a single methyl group on either the 2^{nd} or 3^{rd} carbon were examined (Fig. 6.32-6.33). All assays were performed using Protocol 3 (5.2.1.6).

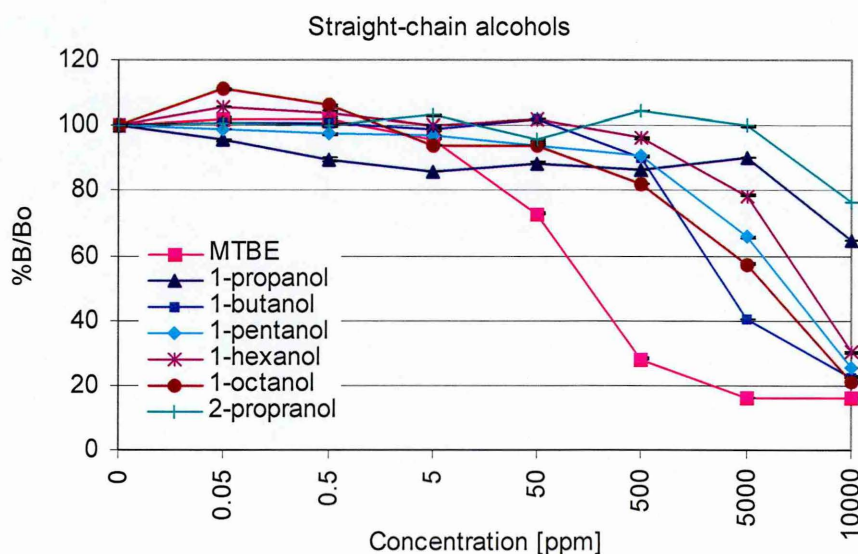


Fig. 6.31 Primary antibody binding on short-chain alcohols

Full-plate assays were conducted with straight-chain alcohols (0-10,000 ppm in PBS). A standard MTBE ELISA was also conducted for comparison (5.2.1.9.2). Results are shown as mean values $\pm$ SD, $n=8$.

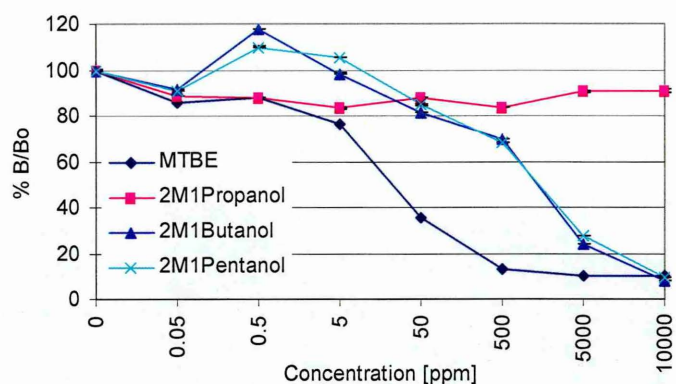


Fig. 6.32 MTBE ELISA response to MODMO analogues (CH_3 group on 2nd carbon)

Straight-chain alcohols (with an additional, single CH_3 group on the 2nd carbon) were assayed alongside MTBE (0-10,000 ppm) using Protocol 3. Results are shown as mean values $\pm$ SD, $n=8$.

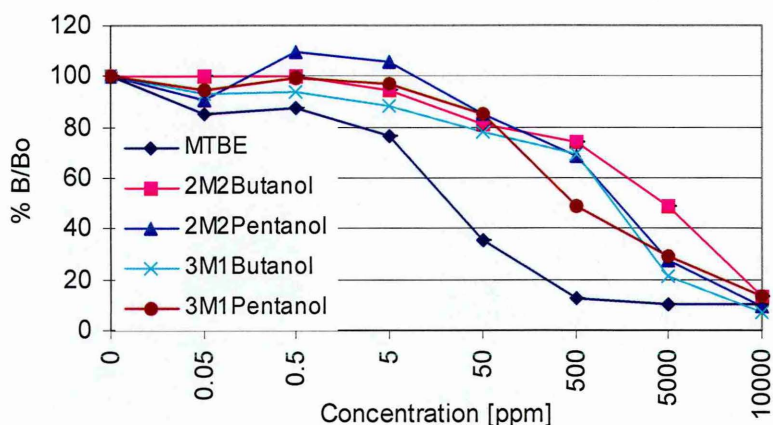


Fig. 6.33 MTBE ELISA response to other MODMO structural analogues

Additional MODMO analogues (5.2.1.9.3) were assayed alongside MTBE (0-10,000 ppm) using Protocol 3. Results are shown as mean values $\pm$ SD, $n=8$.

Results from Fig. 6.31 show that the assay responded to straight-chain alcohols with a minimum of a 4-carbon (*n*-butanol) with the hydroxyl ($-\text{OH}$) group positioned on the 1st carbon. However, higher concentrations of these alcohols (≥ 500 ppm) were required to elicit a response. With 1- and 2-propanol (containing only 3 carbons), concentrations of 10,000 ppm were required. However, this concentration may have interfered with PAb-Ag binding in a more non-specific way (Setford, 2000). Similarly, the A_{450} of the 1-octanol test was 50% lower than the rest of the compounds tested. This is not represented in Fig. 6.31 when displayed as %B/Bo.

In Fig. 6.32, the addition of a single methyl group on the 2nd carbon increased detection of 2-methyl, 1-butanol and 2, methyl 1-pentanol down to <1000 ppm, if taking the 50% B/Bo value. 2-methyl, 1-propanol was not detected, confirming the minimum 4-carbon straight-chain theory.

Greatest sensitivity was to 3-methyl, 1-pentanol (Fig. 6.33), which structurally is very similar to the aldehyde end of the MODMO chain: a 5-carbon straight chain alcohol with a single methyl group on the 3-carbon (Fig. 6.34). The IC₅₀ (%B/B₀) of 3-methyl, 1-pentanol was 500 ppm (compared to 25 ppm MTBE). Placing the –OH group on the 2-carbon did not appear to increase the sensitivity to the other analogues, where the IC₅₀s were ≥2000 ppm.

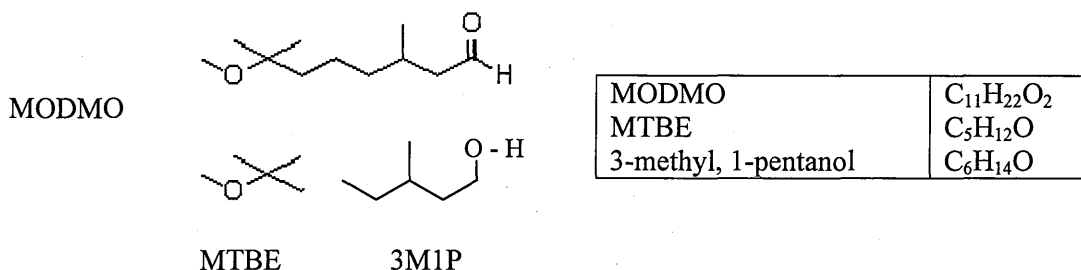


Fig. 6.34 Structures of MTBE, 3-methyl, 1-pentanol and MODMO

MTBE and 3-methyl, 1-pentanol can both be found on either end of the MODMO structure.

6.2.7 Application and validation of the MTBE ELISA

The ELISA performance using real groundwater samples was investigated. Groundwater samples were obtained from monitoring wells throughout Europe (5.1.1.4). Some had been spiked with MTBE, others were naturally contaminated. Details of these sites and any specific groundwater characteristics are detailed within each appropriate method section (5.2.1.10).

6.2.7.1 ELISA performance using MTBE-spiked local groundwater

Natural groundwater (pH 6.7) was obtained from a local, on-site groundwater monitoring well. This was then spiked with MTBE (5.2.1.10) and tested using Protocol 3 (5.2.1.6). Two concentrations ranges were tested simultaneously (Fig. 6.35). Results showed the characteristic sigmoidal curve, with a detection range of 0.5-500 ppm, the IC₅₀ remained at 5 ppm. A further series of experiments examined the effect of buffering the same groundwater to pH 7.4 (Fig. 6.36).

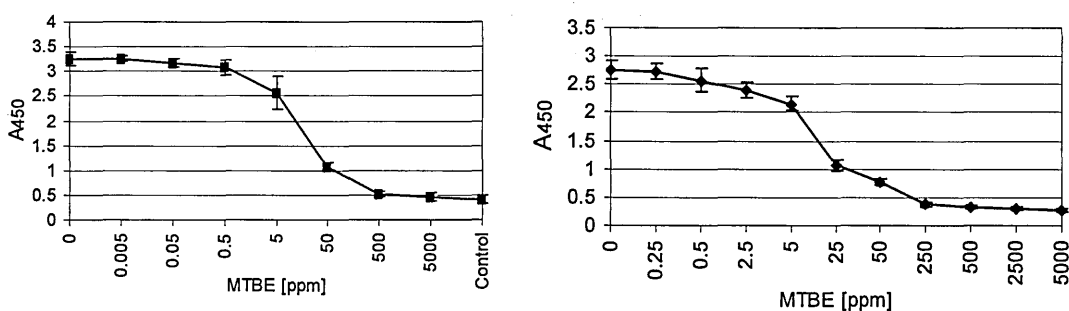


Fig. 6.35 ELISA performance using MTBE-spiked local groundwater

Two full plate assays were conducted using unfiltered, local groundwater (GW) spiked with MTBE (5.2.1.10). The figure on the right shows a more detailed analyte range between 0.25-50 ppm. Mean results are shown ±SD, n=8. The CV was ≤ 8% for both.

A marginal loss in assay sensitivity was found with buffered spiked groundwater solutions, but particularly with PBS (with additional NaCl and KCl salts), which has previously been shown to improve assay repeatability, although at the cost of slightly suppressing assay signal. A large difference in signal was found between unbuffered groundwater solutions and MTBE in PBS.

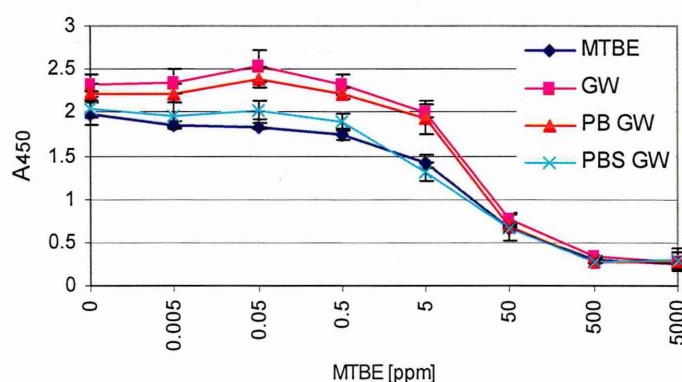


Fig. 6.36 Comparison of buffered and non-buffered MTBE-spiked local groundwater

Two assays were conducted using unfiltered local groundwater (GW) spiked with MTBE and groundwater buffered with PB or PBS (10 mM, pH 7.4, PB GW and PBS GW). MTBE solutions in PBS (10 mM, pH 7.4) were also compared (5.2.1.10). Mean results are shown $\pm$ SD, $n=8$. The CV was $\leq 9\%$ for all assays.

6.2.7.2 ELISA analysis of two Austrian groundwater samples (Salzburg)

In order to assess the potential effectiveness of the MTBE ELISA in different ‘real’ samples, a range of spiked MTBE concentrations were tested in two Austrian groundwater samples, (supplied by Salzburg AG Centre Wasser, Salzburg, Austria) with very different physicochemical parameters (5.2.1.10 and Appendix 4). Two series of MTBE concentrations were made (0-5000, 0-50 ppm) and tested by ELISA (Fig. 6.37). A further series of spiked MTBE concentrations were made which were tested by co-ordinated ELISA and Purge & Trap/GC-MS analysis (6.2.7.2.1).

6.2.7.2.1 GC/MS validation of MTBE ELISA analysis of spiked Austrian groundwater samples

A further series of spiked Austrian groundwater samples were prepared (10, 25, 35, 40 and 60 ppm MTBE). These were divided into 42 ml glass GC vials (two vials per concentration), sealed and stored at 4 °C. Co-ordinated ELISA and Purge & Trap GC/MS analyses were then conducted (5.2.1.10). Fig. 6.38 shows the ELISA results from one set of samples, which were tested twice. These results were then combined and compared to the GC/MS analysis of the second set of samples (Fig. 6.39).

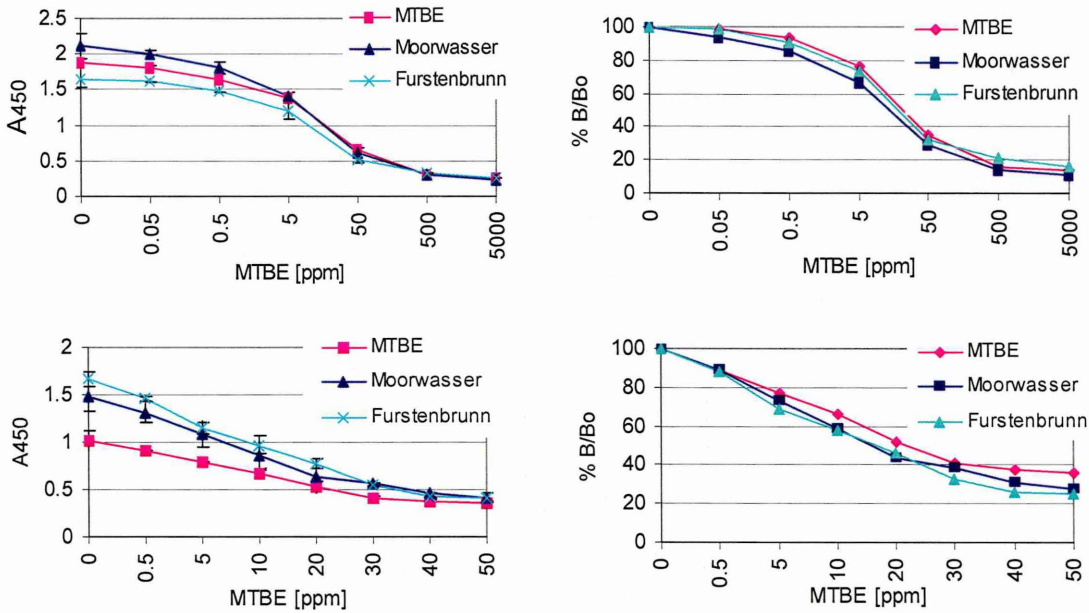


Fig. 6.37 ELISA analysis of two Austrian groundwater spiked with MTBE

Two groundwater samples (Moorwasser and Furstenbrunn) were spiked with MTBE (0-5000 ppm). Further dilutions were made from the 50 ppm samples and are also shown (bottom figures). Standard MTBE solutions (MTBE) were made in PBS. Assays followed Protocol 3. Mean results are shown $\pm$ SD, $n=8$. The CV was $\leq 8\%$ for all assays.

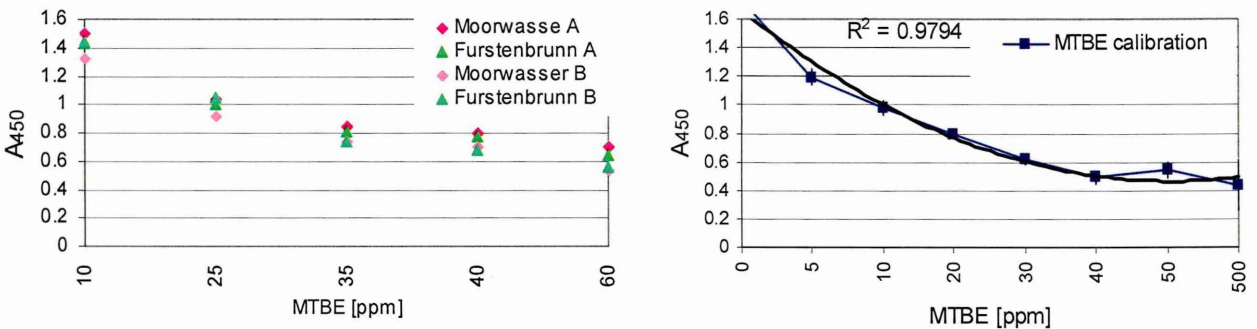


Fig. 6.38 ELISA results of spiked groundwater samples for GC/MS validation

ELISA analysis (of one set of spiked samples) was conducted twice (A and B) following Protocol 3 (5.2.1.6). Mean results are shown $\pm$ SD, $n=8$. The calibration curve (obtained from testing MTBE concentrations in PBS, $n=4$) used to determine final concentrations is shown on the right, $n=2$.

The ELISA results were then combined and compared with the results obtained by GC/MS analysis of the second set of spiked samples (Fig. 6.39). For the ELISA method, the CV was $\leq 10\%$, except for one Moorwasser sample (M60), which was 19%. Both sets of results are compared in Fig. 6.39 and Table 6.1.

Analysis by GC/MS found a general 2-20% difference between the analytical results and the theoretical MTBE concentrations achieved by the original spiking. Higher RSD ($\sim 30\%$) were found in some samples, but this was expected due to the high dilution factors applied (1:50) and

low number of replicates analysed ($n=2$). The difference found by ELISA was 6-18%, with the exception of both F10 and M10 (both 10 ppm), where this increased to 40 and 30% respectively.

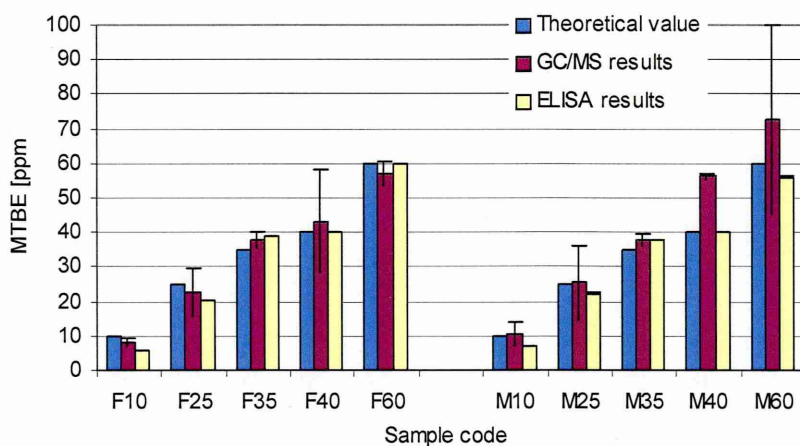


Fig. 6.39 Results of GC/MS and ELISA analysis of spiked Austrian groundwater samples
Two Austrian groundwater samples were spiked with MTBE. The numbers shown in the sample code represent the theoretical values of MTBE concentration in ppm (Table 6.1). GC/MS and ELISA analysis were conducted on the same day (5.2.1.10). GC/MS results are shown as mean values $\pm$ SD, $n=2$, ELISA results are shown as mean values $\pm$ SD, $n=16$. Actual values are provided in Table 6.1.

Table 6.1 Results from GC/MS and ELISA analysis of spiked Austrian groundwater samples

Two Austrian groundwater samples were spiked with MTBE. GC/MS and ELISA analysis was conducted on the same day. Results are shown plotted in Fig. 6.39.

Sample code	Theoretical value [ppm]	P&T-GC/MS [ppm] $n=2$	SD	CV (%)	ELISA [ppm] $n=16$	SD	CV (%)
F10	10	8.19	0.96	11.77	6.00	0.004	0.29
F25	25	22.72	6.99	30.77	20.40	0.032	3.13
F35	35	37.66	2.41	6.41	39.00	0.050	6.49
F40	40	43.28	14.77	34.12	40.00	0.061	8.34
F60	60	56.98	3.43	6.02	59.92	0.061	10.18
M10	10	10.46	3.64	34.74	7.00	0.132	9.34
M25	25	25.44	10.78	42.35	22.40	0.086	8.83
M35	35	37.86	1.53	4.04	37.60	0.080	10.07
M40	40	56.13	1.04	1.84	40.00	0.064	8.43
M60	60	72.73	27.40	37.68	56.00	0.120	19.14

6.2.7.3 Analysis of natural MTBE-contaminated groundwater samples, Dresden, Germany

MTBE and BTEX-contaminated groundwater samples were obtained from Dresden, Germany (5.2.1.10.3). Samples had been obtained via underwater motor pumps from 3 wells 200 m

downstream of a MTBE refinery site in Leuna, Dresden. Samples were tested independently by both the MTBE ELISA (Cranfield University) and GC/FID headspace analysis (Dresden University). Fig. 6.40 shows good correlation between the two detection methods. BTEX contents (determined by GC/FID) were reported as ~30 ppb in samples *saf38* and *leu*. These samples also contained TBA at 0.9 and 0.5 ppm respectively. TBA would have been detected and reported as ‘MTBE’ using the ELISA, as this compound has been previously shown to cross-react (Fig. 6.28). This is an advantage with the MTBE ELISA, as TBA (the natural breakdown product of MTBE) is often not tested for and so by reporting MTBE/TBA as ‘total’ MTBE, the MTBE ELISA can provide a more complete picture of MTBE contamination and evidence of a historic MTBE contamination.

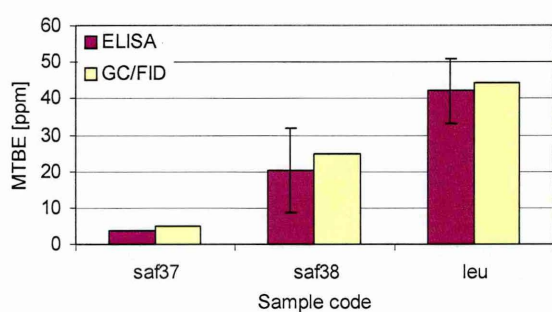


Fig. 6.40 Results of ELISA and GC/FID headspace analysis of MTBE-contaminated groundwater

Dresden groundwater samples were serially diluted (1:2) across plate wells and concentrations determined from a MTBE calibration curve (5.2.1.10.3). ELISA results are shown as mean values $\pm$ SD, $n=3$. The assay was performed as Protocol 3. GC/FID results shown are as reported by Dresden University.

6.2.7.4 Koblenz field-testing and independent validation of the MTBE ELISA

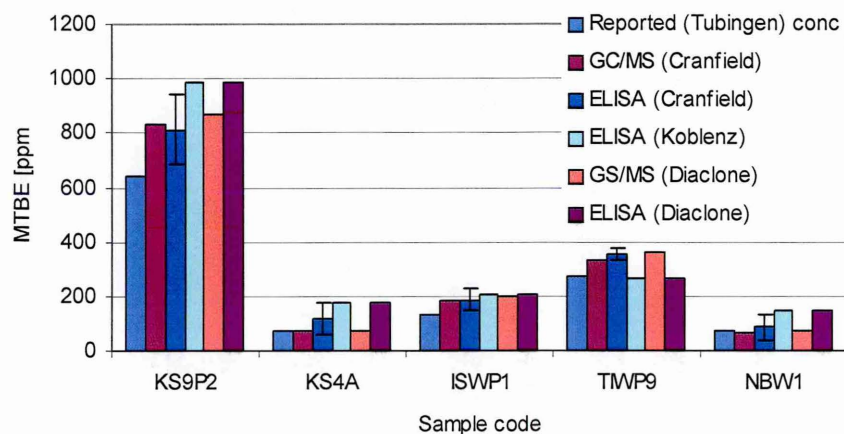
The MTBE ELISA was validated with field testing in Koblenz, Germany as part of SENSPOL Technical Meeting, Koblenz, Germany (5.1.1.4). Water samples were taken from various sites and spiked with MTBE and BTEX compounds (5.2.1.10.4). The MTBE ELISA was conducted on-site by a hydrologist operator, who had received only brief training in ELISA techniques, his results were subsequently validated independently by 3 groups: Cranfield (ELISA), Diaclone SAS (ELISA) and CSIC (GC/MS). These results are shown in Table 6.2 and plotted in Fig. 6.41.

Fig. 6.41 shows the results from the field trials and the independent analysis (ELISA and GC/MS). The effect of sample filtration was also investigated (Fig. 6.42). Table 6.3 shows the BTEX content of samples as determined using the Total BTEX RaPID immunoassay test kit (Chapter 1, Section 1.5.2.2), following the manufacturer’s protocol. GC/MS analysis for BTEX was also conducted by CSIC (Barcelona, Spain).

Table 6.2 Results of multiple ELISA analysis of Koblenz water samples

Duplicate aliquots of each unknown sample were diluted (1:2) across plate wells (8 dilutions). Mean absorbance values were taken and plotted against the calibration curve and the MTBE concentration read off the x-axis and multiplied by the dilution factor, resulting in eight values per sample. The mean values displayed in the table were obtained from points falling within the OD range of 0.6-1.7, equating to the assay sensitivity range of 5-500 ppm, which correlates to the more linear part of the sigmoidal response curve. This was done seven times for each water sample. The mean of the seven assays is shown in blue, with the appropriate SD and CV of these assays. Results (ELISA and GC/MS) from the other participating parties are also shown.

Cranfield ELISA	Sample code				
No. of full plate assays	KS9P2	KS4A	ISWP1	TIWP9	NBW1
1	880	80	120	385	40
2	700	20	150	315	20
3	645	82	177	322	94
4	1072	191	251	364	124
5	812	194	174	385	158
6	808	136	214	353	109
7	766	141	214	350	50
Cranfield Mean	812	121	186	353	85
<i>Cranfield SD</i>	<i>128.0563</i>	<i>58.86512</i>	<i>40.82966</i>	<i>25.5838</i>	<i>46.26322</i>
<i>Cranfield CV</i>	<i>15.77325</i>	<i>48.82178</i>	<i>21.9852</i>	<i>7.238746</i>	<i>54.42732</i>
GC/MS (Cranfield samples)	831	70	182	328	69
Reported conc. (Tübingen)	638	74	135	272	74
ELISA (Koblenz field trial)	986	180	203	267	148
ELISA (Diacclone SAS)	986	180	203	267	148
GS/MS (Diacclone samples)	872	72	202	361	76

**Fig. 6.41 Field trial results and independent assay validation using the MTBE ELISA**

Collected water samples were spiked with MTBE and tested by ELISA on-site. Independent ELISAs and GC/MS analysis were performed by 3 independent groups (5.2.1.10.4). Cranfield results are shown as mean values $\pm$ SD ($n=7$), obtained from full-plate assays.

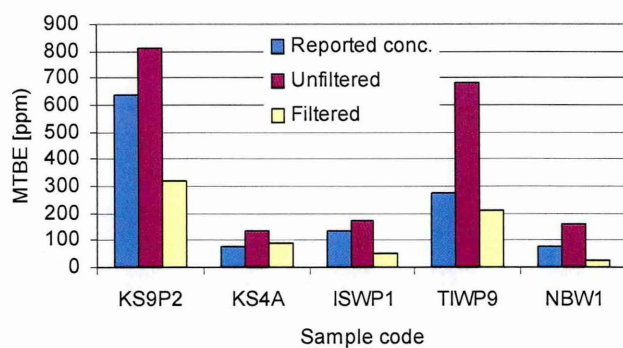


Fig. 6.42 Effects of filtration of Koblenz water samples for analysis by ELISA

Koblenz water samples were passed through a 5 μm filter (5.2.1.10). Assays were conducted with both filtered and unfiltered samples following Protocol 3 (5.2.1.6). ELISA results are shown as mean values, $\pm\text{SD}$, $n=8$, compared to the reported MTBE concentrations.

Table 6.3 Cranfield ELISA and GC/MS results for Total BTEX

Water samples supplied from Koblenz were tested for Total BTEX by ELISA and independent GC/MS analysis by CSIC (5.2.1.10.4). Results for ELISA are shown as mean values, $\pm\text{SD}$, $n=2$.

Sample name	Sample ID	Reported BTEX & PAH [ppm]	ELISA [ppb] ¹	GC/MS [ppb] ²
Kiel Canal S9 P2	KS9P2	2-2000	700	2609
Kiel Canal S4A	KS4A	2-2000	700	518
Iffezheim Surface Water P1	ISWP1	-	190	529
Tiefer See Interface Water P3	TIWP3	-	210	533
Niederworth BW1	N-BW1	-	120	536

¹ Tübingen University data

² Cranfield University, Silsoe

³ CSIC, Barcelona

6.3 CONCLUSIONS AND DISCUSSION

Pilot assays indicated that the PAb bound only to the BSA-MODMO Ag conjugate. Whilst these assays could clearly also detect free MTBE, there was no apparent binding to BSA-MTBE Ag. This was not altogether unexpected for two primary reasons: (1) the PAb had been raised against the BSA-MODMO Ag and (2) it is difficult to envisage how free MTBE, being a relatively unreactive molecule, can be linked to the BSA carrier protein. Indeed, the success of the Diaclone BSA-MTBE conjugation is interesting given the fact that the conjugate did not produce an immunogenic response in mice (Diaclone SAS, *pers.comm*).

Optimisations of the preliminary ELISAs

Early optimisation steps determined that the PAb was quite specific to BSA-MODMO Ag and that free PAb did not bind to free BSA protein in solution. BSA could therefore be used as the blocking buffer. Later experiments found that 1% BSA (w/v) was sufficient for blocking Ag-coated plates, higher concentrations producing no effective decrease in NSB (Fig. 6.3).

Optimal Ag-coating concentration was found to be 500 ng/ml (50 ng/well). Using biotinylated PAb and s-HRP (Protocol 1, 5.2.1.2) required an optimum PAb concentration of 100 ng/well. Using the native PAb/2Ab-HRP combination allowed a 50% reduction in PAb usage (Protocol 3, 5.2.1.6), as only 50 ng/well gave improved assay signals, indicative of improved PAb-Ag binding and possibly more effective PAb capture of the 2Ab-HRP.

Unfortunately, combining the primary and secondary incubation steps caused a drastic loss of signal with both biotinylated and native PAb (95 and 40% respectively). Although sensitivity appeared to remain about the same using the latter method, the benefits of increased assay signals outweighed the benefit of a 30 min reduction in assay incubation times. Clearly, the HRP label interfered with PAb-Ag binding.

Additional advantages of using native PAb are as follows:

- The biotinylation step (performed by the PAb supplier, Diaclone SAS) can be avoided. Secondary detection of the immobilised PAb-Ag complex is achieved through using commercially available 2Ab-HRP conjugate.
- Using native PAb and 2Ab-HRP gave more consistent and reproducible results, with higher overall assay signals.
- A great deal of variability was found between two separate s-HRP products (Fig. 6.13) which could ultimately impact the overall sensitivity and accuracy of the assay.
- Long-term storage of s-HRP (at -20 °C) was not recommended by either supplier, whereas both PAb and 2Ab-HRP can be aliquoted and stored long-term at this temperature. Once thawed, these smaller aliquots can be stored at 4 °C without any significant decrease in performance after 1 month.
- The native PAb can be expected to have greater specificity and affinity than a derivatised (biotinylated) PAb, as conjugations can alter the natural protein structure, potentially reducing the number of available Ag binding sites and/or reducing the avidity of Ag capture. It is interesting to note that the preferred PAb/2Ab-HRP system had been subjected to a single conjugation chemistry, whereas the biotin/streptavidin-HRP system had endured two.

Choice of other reagents and assay conditions

Buffers: Early results seemed to suggest that PAb-Ag binding was better in RO water (pH 6.7), rather than in buffer (PBS) as signals were higher overall. It was found that increased ionic strength (PBS concentration) during the initial PAb-Ag binding step depressed assay signals,

presumably from an inhibitory salt effect. However, assay sensitivity and reproducibility were better using a 10 mM PBS solution when diluting the PAb. This is effectively diluted by 50% during the PAb-Ag binding when using real water samples. The 2Ab-HRP bound perfectly well in 10 mM PBS during the second incubation step. When other buffers (Tris and PB) replaced PBS (at the same concentration), they did not suppress the assay signals as much, but greater inter-well variations were found in terms of assay result. Experiments were also conducted to check the linearity of the colour development (5.2.1.3.4), which was linear when the PAb-Ag binding was conducted in PBS.

TMB based colour substrate: Colour development (due to HRP activity) with the TMB substrate solution results in a blue colour, which is read at 630 nm. Reaction can be stopped after fixed times by addition of Stop solution (H_2SO_4); in this case, the colour is yellow and absorbance is read at 450nm. Acidification gives the largest signals. Three different commercial TMB substrates were used in this work and they gave very different results. Assay performance with the 'Ultrasensitive' product from Europa greatly surpassed the other two brands. The precise reasons for this are difficult to ascertain. All of these products are basically different preparations of the fundamental HRP assay cocktail, which is hydrogen peroxide, oxidisable substrate (in this case TMB), buffer and perhaps some solution stabiliser. The pH and concentration may well vary and this is probably the root of the differences in performance. However, because of commercial propriety, the precise details of each substrate are not publicly available.

The durability or 'shelf-life' of the reagents used in the MTBE ELISA needs to be determined further. For example, investigating how many times the reagents (PAb and Ag) be brought from 4 °C to room temperature, used and re-stored (at 4 °C) before significant denaturing occurs, consequently reducing the efficiency of the assay. It has been shown that some commercial reagents (such as s-HRP conjugate) deteriorate rapidly, possibly skewing results.

Assay responses increased when a higher temperature (27 °C) was used for the Ab-binding steps. At 37 °C, results were erratic, most probably due to changes in the conformity of the enzyme, resulting in less efficient catalyse (temperatures >40 °C denature most mammalian enzymes, Tijssen, 1993). Possibly, MTBE evaporation also increased. As the assay is designed for use in the field, an ambient temperature of ~22 °C is perhaps more practicable. However, any variation from this may have an impact on assay signal and consequent reproducibility.

A pH of 7.4 was shown to be most suitable for the MTBE ELISA and for diluting the antibodies. All real water samples tested fell with a pH range of 6.7-7.4 and were brought to pH

7.4 by buffering with PBS tablets. Extreme pH values of 4 and 9 drastically reduced the assay signal, most likely through interfering with PAb-Ag binding, or perhaps denaturing the immunoreagents. Indeed, BSA is known to change configuration at these pHs and this could impact the positioning and subsequent availability of the antigenic site on the BSA-MODMO conjugate.

Stability of MTBE solutions

Under normal conditions, MTBE is a clear liquid with a characteristic pungent smell. It has a density of 0.74 g/ml and is miscible with water. Its boiling point is 55 °C, this compares to 56 °C for acetone and 69 °C for hexane, and thus it is very volatile. This initially led to concerns that MTBE solution (or real samples) would not be stable, in that concentrations would decrease due to loss to the atmosphere. However, experiments with 'aged' solutions of MTBE in PBS (Fig. 6.24-6.25) indicated that this was not the case. Even with forced evaporation (by vigorous nitrogen bubbling) significant MTBE loss only became apparent after 1 hr (Fig. 6.24). During the course of these experiments, the unexpected observation was made that ELISA determination of freshly-made MTBE solutions were quite variable, but became less so after a 1-2 hours. This is believed to be due to there being a finite time required for complete MTBE dissolution (up to 3 hr), an idea which is supported by the observation of a surface film, which forms on MTBE addition (in excess of 10,000 ppm) to aqueous samples and which subsequently disappears. When known MTBE solutions were made in RO water or PB (rather than PBS), it was noted that greater inter-well variation occurred in the ELISA result, particularly at the lower concentrations. This led to the interesting speculation that perhaps the salt content in PBS stabilised the solutions by decreasing MTBE volatility.

Detection of other fuel oxygenates (cross-reactivity)

The MTBE ELISA can also be used to measure the following fuel oxygenates; TAME, DIPE, ETBE (in order of decreasing sensitivity). It can also detect TBA and TBF (Figs. 6.27-6.28). TBA is always found as an impurity in MTBE and both TBA and TBF are natural biological breakdown products of MTBE (Ray *et al.*, 2003). As a result, MTBE contamination can be 'under-detected' as a result of natural biodegradation and/or lack of testing for TBA and TBF (Squillace *et al.*, 1995; US EPA 1997b). The MTBE ELISA overcomes this by reporting all three compounds (MTBE, TBA, TBF) as 'MTBE'. Similarly, oxygenates are not usually added to diesel fuel or central heating oil, consequently the MTBE ELISA can be used to identify an environmental fuel spill as petroleum. Similarly, the US EPA methods #8020/8021 (incorporating PID) for testing fuel oxygenates (MTBE, ETBE, DIPE and TAME) have been found to be strongly affected through interference by petroleum hydrocarbons (≥ 500 ppb TPH)

and resulted in false-positives and false negatives for oxygenate identifications (Happel *et al.*, 1998). Method #8020 is indeed no longer recognised by the EPA (API, 2000). There was no evidence of interference from BTEX compounds with the MTBE ELISA in groundwater samples (provided by Dresden University).

Cross-reactivity with other organic compounds

As expected, the MTBE ELISA displays a degree of cross-reactivity with organic compounds that have some structural analogy to MTBE. Examples investigated in this study are straight chain alcohols, which cross-react from a minimum chain length of 4 carbons (1-butanol) up to the 8 carbon (1-octanol), the longest tested (Fig. 6.26). Sensitivity to these alcohols is increased when an additional methyl group is positioned on the 3-carbon and was most notable in the case of 3-methyl, 1-pentanol (Fig's. 6.31-6.33). This is perhaps not surprising, as this compound is structurally very similar to the 'aldehyde end' of 7-methoxy-3, 7 dimethyloctanal (MODMO), which is the actual target analyte the PAb was raised against (Fig. 6.34). The cross-reactivity of 3-methyl, 1-pentanol is important in that it emphasises the fact that the 'MTBE' ELISA developed during this work is perhaps really a 'MODMO' ELISA (in terms of the PAb binding) and this is reflected in the very large difference (1000 x) in assay sensitivity to the two compounds.

Sensitivity of the MTBE ELISA

The assay sensitivity range (linear portion of the sigmoidal curve) for MTBE remains between 5-500 ppm, with the mid-range sensitivity (IC₅₀) around 5 ppm, well above the average drinking water quality standards of 15-30 ppb. However, sensitivity to MODMO was found to be around 5 ppb, which calculates as a 1,000-fold difference in sensitivity.

Assay validation with 'real' water samples

The MTBE ELISA was validated through testing a number of real groundwater samples, some of which were naturally contaminated (as opposed to being spiked with authentic MTBE). Assays conducted in unbuffered, spiked local groundwater samples generally produced signals, which tended to be higher than those of the equivalent MTBE standards, which were made in PBS. Buffering the groundwater resolved this phenomenon.

Results from the testing of spiked Austrian groundwaters (Fig. 6.37-6.38) correlated well with independent GC/MS analysis. Although the physico-chemical parameters of these two groundwaters were different (namely cation/anion content), this had no adverse influence on the assay performance. Good correlation between the MTBE ELISA and GC/FID was also found in naturally contaminated groundwaters from Dresden, Germany.

Blind field test results by an unpractised operator generally over-estimated MTBE in spiked river water samples. Extensive post-sampling testing at Cranfield also over-estimated MTBE but not to such a degree. This is possibly attributed to the presence of other cross-reactive compounds, which were not looked for on the GC. A suggested 96-well plate assay format is given in Fig. 6.44. Fine filtration of samples resulted in a loss of sensitivity, most likely through MTBE evaporation during the filtering step or possibly adsorption to the filter material.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	S	S		S		S		S		M	
B	1:2	A	A		A		A		A		T	5000
C	1:4	M	M		M		M		M		B	500
D	1:8	P	P		P		P		P		E	50
E	1:16	L	L		L		L		L			5
F	1:32	E	E		E		E		E		P	0.5
G	1:64										P	0.05
H		A	B		C		D		E		M	0
		↓		↓		↓		↓		↓		
		No PAb controls (alternatively, no Ag controls)										

Fig. 6.43 Suggested field 'blind' testing assay format using a 96-well plate

Duplicate dilutions of 5 different unknown water samples are shown in Col. 1. Duplicate MTBE concentrations (in PBS) are shown in Col. 11-12 for calibration curve; these also act as positive controls. The final row (H) should be used for controls lacking PAb in each water sample.

Summary

This chapter has described the development of a novel, competitive ELISA for MTBE. The work has followed the course of a rigorous optimisation of immunoassay conditions with regard to:

- Plate immobilised Ag conjugate
- Requirement for blocking agent
- Reagent formulation (concentrations, pH, salt and buffer contents)
- Incubation times and conditions (agitation, temperature)
- Method of label (HRP) binding (PAb/2Ab-HRP or b-PAb/s-HRP)
- Selection of commercial colour substrate solution

This work has resulted in an assay, which features Ag (BSA-MODMO) immobilisation, followed by PAb/MTBE incubation and then 2Ab-HRP. The assay takes 65 minutes and has a IC₅₀ around 5 ppm. Using a single 96-well plate, 5 different water samples can be tested in duplicate. The assay can easily be performed in the field by a relatively, unskilled operator, the equipment required being simply a portable plate reader, plate washer, pipettes and a coldbox for immunoreagents.

The assay IC₅₀ of 5 ppm MTBE is above the average drinking water quality standard of 15 ppb. However, the assay was developed primarily as a rapid field-based screening tool for evaluation of the extent and migration of a new fuel pollution incident, where MTBE concentrations would initially be very high. Indeed, an IC₅₀ is comparable to other commercial IAs, such as the RaPID Total BTEX assay (SDI Inc.) has a limited sensitivity range of only 0.09-3 ppm. In fact, for the more soluble benzene component, the test has a limit of quantification (LOQ) of 7.1 ppm in water samples and rises to an LOQ of 70.1 ppm (after methanol-based solvent extraction using another of their commercial testing kits). The commercial assay cannot differentiate between related compounds and cross-reacts with 20-30 other compounds.

The MTBE assay sensitivity is limited, most probably due to the small molecular weight (88 Da.) or more precisely, small molecular size, of MTBE. This results in the fact that it is only able to establish relatively few binding interactions in the (much larger) Ag-Ab binding site, which is 'designed' to recognise MODMO. In this particular case, this has clearly resulted in the MTBE binding with quite low affinity, necessitating high concentrations for a significant competitive inhibition effect on the Ag-Ab interaction. It could be argued that an antibody with improved MTBE binding characteristics would improve the assay. However, it is probable that only a limited improvement would be seen given the small size of the MTBE molecule. Indeed, some 3,000 hybridomas were screened (Diaclone SAS) in order to obtain a single PAb sensitive to MTBE.

The PAb preferentially binds to part of the MODMO structure, as this was what it was raised against. However, MODMO is conjugated to BSA via its carbon chain and terminal aldehyde. Therefore, the remaining MODMO structure 'seen' by the PAb looks the same as MTBE bound to an additional 5 carbon chain. As shown with the structural analogues (6.2.6), this is essentially very similar to 3-methyl, 1-pentanol. The antibody FAB fragments most probably binds to this as well as the MTBE moiety. MTBE is a rather small molecule (88 Da.) and so can only occupy a small proportion of PAb recognition sites.

In terms of applicability for field testing and the number of water samples which can be testing in a relative short time, the MTBE assay has proved to be a reliable, rapid and cost effective screening method of determining MTBE contamination. In light of these findings, alternative assay formats were investigated in order to improve and simplify field-testing methodologies and are reported in the following sections.

6.3 PROTEIN CONJUGATIONS

An important part of competitive immunoassay development is the covalent linkage of low molecular weight haptens (the 'target' analyte, or a structurally related analogue) to a protein, for example, the BSA-MODMO antigen conjugate. Preparation of this conjugate usually involves two processes (1) modification of the carrier protein by the addition of linking groups and (2) combining these groups with the target to produce the complete conjugate. In this work, the BSA-MODMO conjugate was also used as the immunogen in order to generate the antibody response in the host animal.

The optimised immunoassay developed in this work was based on the competitive immunoassay format with the BSA-MODMO antigen conjugate (Ag) immobilised in microtitre wells. By adding a sample containing free MTBE along with primary antibody, it was found that MTBE competed with the immobilised Ag for PAb binding sites. This assay format (illustrated in Chapter 1, Fig. 1.8) required a secondary antibody, which bound to the PAb and was conjugated to a detection label, such as streptavidin-HRP (if using a biotinylated PAb) or goat anti-IgG mouse-HRP (if using native PAb). The addition of an HRP colour substrate, such as TMB, would serve to identify the proportion of captured BSA-MODMO antigen and hence free analyte present in the original sample. High colour would result from maximal BSA-MODMO binding, indicating no MTBE present within the sample tested. Conversely, minimum colour would indicate a high MTBE content in the sample.

An alternative competition assay format could be to have the PAb immobilised onto the well surface (Fig. 6.44). By adding a sample containing free MTBE and either a BSA-MODMO-HRP conjugate (or perhaps MODMO-HRP, assuming some cross-reactivity with MODMO-BSA), direct competition for PAb binding sites could be achieved, removing the need for a secondary Ab/ligand labelling step. This would effectively simplify the assay procedure and reduce overall assay time, both advantageous for field use. These conjugated molecules could also aid transposing the assay into other formats (Sections 6.4-6.5). For antigen labelling, two investigative conjugations were made and tested with immobilised PAb in microtitre plate wells (5.2.2.2):

- Combination of MODMO directly to the HRP enzyme
- Combination of the BSA-MODMO antigen to HRP

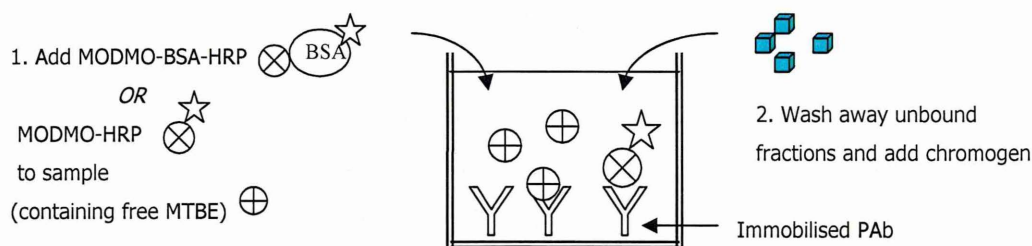


Fig. 6.44 Immobilised PAb assay format

The PAb is immobilised onto the microtitre well. Free MTBE (if present) in the water sample will compete for PAb binding sites with a known quantity of MODMO-HRP conjugate (or the MODMO-BSA-HRP conjugate). After incubation, unbound fractions are removed by washing. The chromogen (TMB) is then added to the well and will be catalysed by HRP (bound MODMO-HRP or MODMO-BSA-HRP). Colour development would be inversely proportional to the concentration of MTBE in the sample (low colour indicating high MTBE).

An alternative could be to bind the primary antibody directly to HRP, which would effectively change the ELISA format to a straightforward ‘single-step’ detection method. Direct labelling of PAb is sometimes a favourable option as there is generally less background noise and non-specific binding. However, labelling PAb involves a conjugation step, followed by separation, usually by affinity chromatography, or alternatively by dialysis. A disadvantage of ‘self-labelling’ is the potential to waste limited supplies of expensive PAb and other reagents, with no guarantee of success. In some cases, such as with low levels of analyte, some form of signal amplification is also required, usually by employing a secondary labelled polyclonal Ab. As the MTBE ELISA has already shown itself to be relatively insensitive ($IC_{50} \sim 5$ ppm), this route was not taken.

6.3.1 Determination of the MODMO aldehyde group activity

The first step was to confirm that the aldehyde group (CHO) on the MODMO compound was an active binding group. This was determined by using a Glycoprotein Detection Reagent (GDR, 5.1.2.2), which is used to detect aldehydes after oxidation of glycoproteins with sodium *meta*-periodate ($NaIO_4$). The aldehyde formed reacts with the GDR and produces a purple colour. The absorbance is proportional to the percentage of carbohydrate components present. However, the aldehyde group should already be present on the MODMO compound, therefore the reaction with the GDR and the subsequent colour would be a confirmation that the oxidation to carboxylic acid had not occurred. The test was performed with and without the oxidation step and the high colour produced (Fig. 6.45) confirmed the binding activity of the aldehyde group.

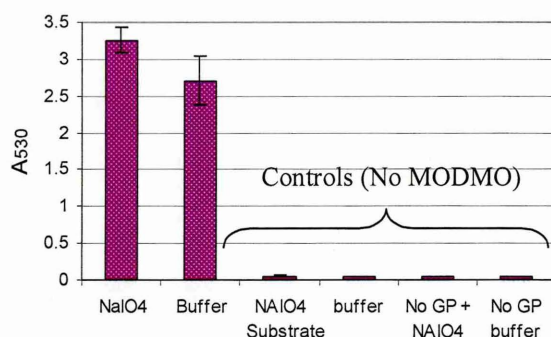


Fig. 6.45 Determination of the MODMO aldehyde group activity

The activity of the MODMO aldehyde group (CHO) was determined using a Glycoprotein (aldehyde) Detection Reagent (5.1.2.2). The oxidised test (with NaIO₄) is labelled as NaIO₄, the unoxidised test is labelled 'buffer'. Controls lacked MODMO. Results are shown as mean values, +SD, n=6.

6.3.2 Direct conjugation of MODMO to HRP

HRP (MW 42,000) has 6 lysine residues per molecule, but only 2 are generally available for conjugation and these supply the primary amine groups (-NH₂) needed to bind the MODMO aldehyde groups (CHO). Therefore a minimum of 2 mM MODMO would be required to coat 10 mM HRP. To ensure maximum binding, MODMO was applied in excess (5.2.2.2). The aldehyde reacts with the primary amines on HRP to form a Schiff base (CHNH), followed by reduction of the imine by sodium cyanoborohydride (reducing agent). The amine nitrogen launches a nucleophilic attack on the electrophilic carbonyl group, this is followed by a dehydration across the C-N bond to form the Schiff's base. The borohydride provides hydride ions, which reduce the double bond and stabilises the protein-ligand bond. The Schiff base structure is shown in Fig. 6.46. Method developments are detailed in Chapter 5 (Table 5.6).

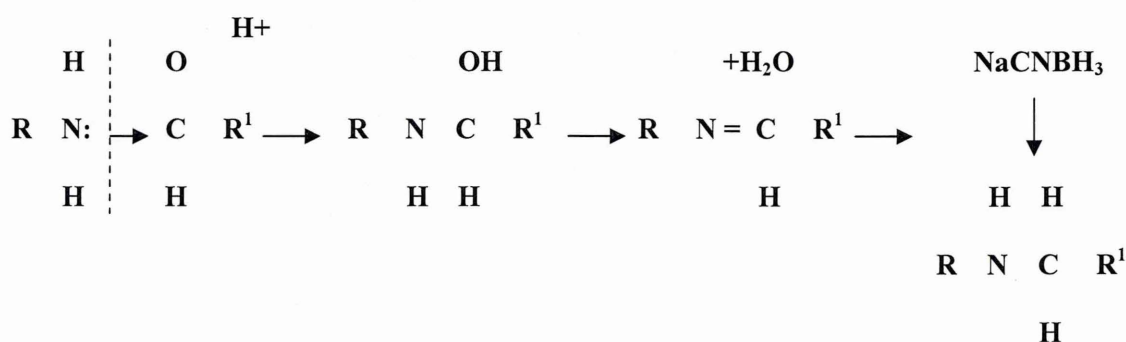


Fig. 6.46 Schiff base conjugation principle

The N donates 2 electrons to the carbon on the aldehyde group, which cleaves the double bond binding the oxygen, forming a single bond with a hydrogen ion. A water molecule is expelled, so the aldehyde becomes bound to the protein by a C=N- bond. This structure is stabilised by the addition of a reducing agent (NaCNBH₃), to form a stable C-N single bond.

6.3.3 Assay responses with MOMDO-HRP conjugates

Four separate MODMO-HRP conjugations were prepared with minor variations between each conjugation method (5.2.2.2). These conjugates were then tested in a series of microtitre plate assays using blocked (Fig. 6.47) and unblocked (Fig. 6.48) PAb-coated plates (5.2.2.3), with and without MTBE present (5000 ppm in PBS). Fig. 6.49 shows results from conjugation methods 3 and 4, where the conjugation protocol had been refined further and subsequently tested at two temperatures (5.2.2.3.3). In this latter experiment, no MTBE was present, as the aim here was to increase assay signals.

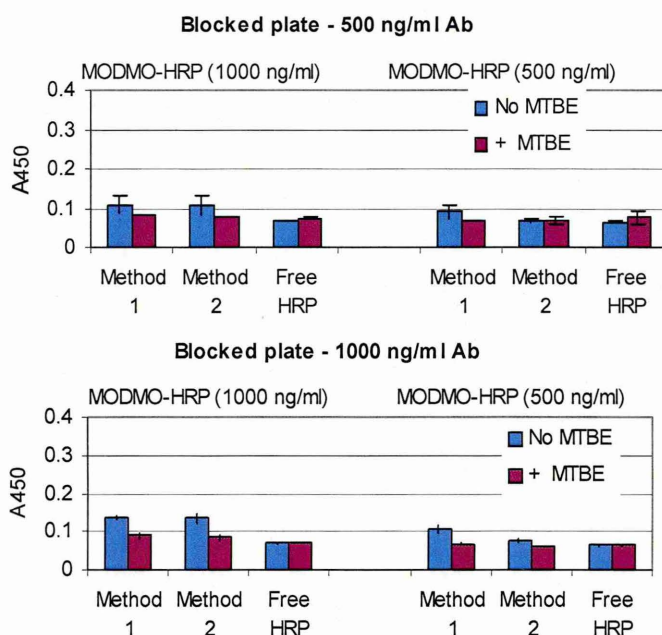


Fig. 6.47 Assay response with MODMO-HRP conjugates

Results show the assay responses from MODMO-HRP conjugates prepared using conjugation methods 1 & 2 (5.2.2.2). Plates were coated with two concentrations of primary antibody (500 & 1000 ng/ml) and blocked with 1 % BSA (w/v). The MODMO-HRP was tested at two concentrations (500 & 1000 ng/ml). MTBE was present at 5000 ppm (in PBS), where indicated. Results are shown as mean values, $\pm$ SD, $n=4$.

These early results showed that some distinction could be made between samples with or without MTBE (Fig. 6.47) although the overall assay response of ~ 0.1 (A450) was low. Assays conducted on unblocked plates (Fig. 6.48) showed a two-fold signal increase with the higher concentration of MODMO-HRP (1000 ng/ml), however, distinction between samples were inconsistent. Higher signals (~ 0.4 A450) were achieved with MODMO-HRP conjugations made using Methods 3 & 4 (Fig. 6.49) with 1000 ng/ml PAb and MODMO-HRP concentrations. These results are discussed further in Section 6.3.7.

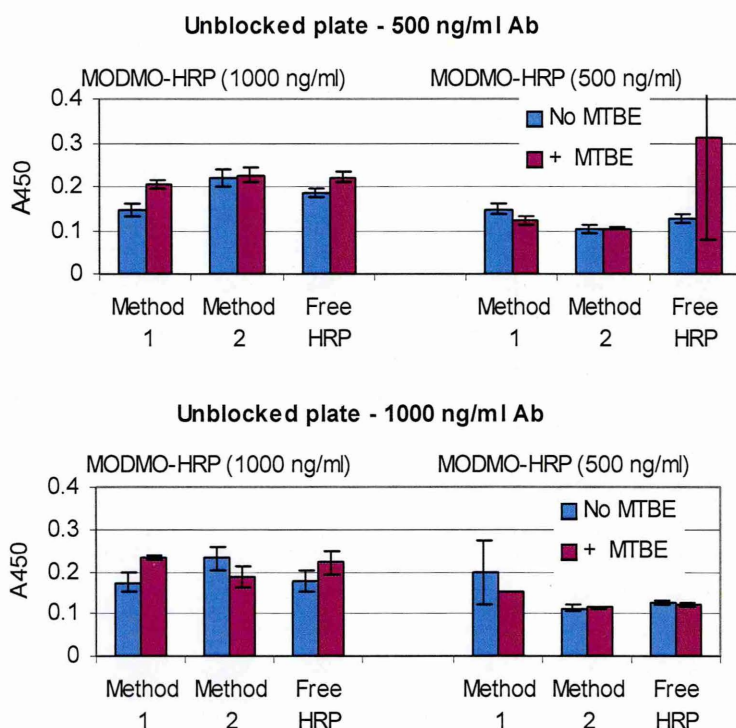


Fig. 6.48 Assay response with MODMO-HRP conjugates

Results show the assay responses from MODMO-HRP conjugates prepared using conjugation Methods 1 & 2 (5.2.2.2). Plates were coated with two concentrations of primary antibody (500 & 1000 ng/ml) and left unblocked (5.2.2.3). The MODMO-HRP was tested at two concentrations (500 & 1000 ng/ml). MTBE was present at 5000 ppm, where indicated. Results are shown as mean values, $\pm$ SD, $n=4$.

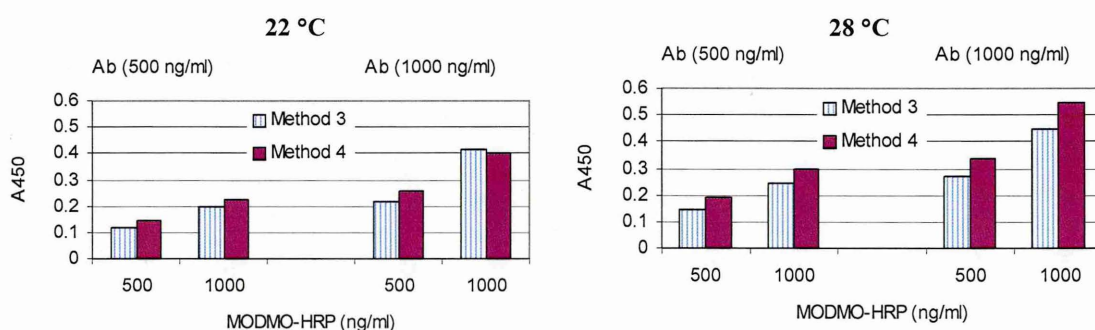


Fig. 6.49 Assay response with MODMO-HRP conjugates

Results show the assay responses from MODMO-HRP conjugates prepared using conjugation methods 3 & 4 (5.2.2.2). Plates were coated with two concentrations of primary antibody (500 & 1000 ng/ml) and blocked with 1 % BSA. The MODMO-HRP was tested at two concentrations (500 & 1000 ng/ml) and incubated at either 22 or 28 °C (5.2.2.3.3). Results are shown as mean values, $\pm$ SD, $n=4$.

6.3.4 Conjugation of BSA-MODMO antigen complex with HRP

The BSA-MODMO-HRP conjugate was diluted to 500 and 1000 ng/ml and was tested in blocked and unblocked PAb-coated plates (Figs.6.50-6.51), assays were conducted as for the MODMO-HRP conjugates; the MODMO-HRP conjugate was merely replaced with BSA-MODMO-HRP conjugate (5.2.2.3). A checkerboard assay was also performed (Fig. 6.52) using a microtitre plate coated with a range of native PAb concentrations and blocked with 1% BSA (w/v). The BSA-MODMO-HRP conjugate was diluted in PBS to give a concentration range of 125-1000 ng/ml (5.2.2.5.1).

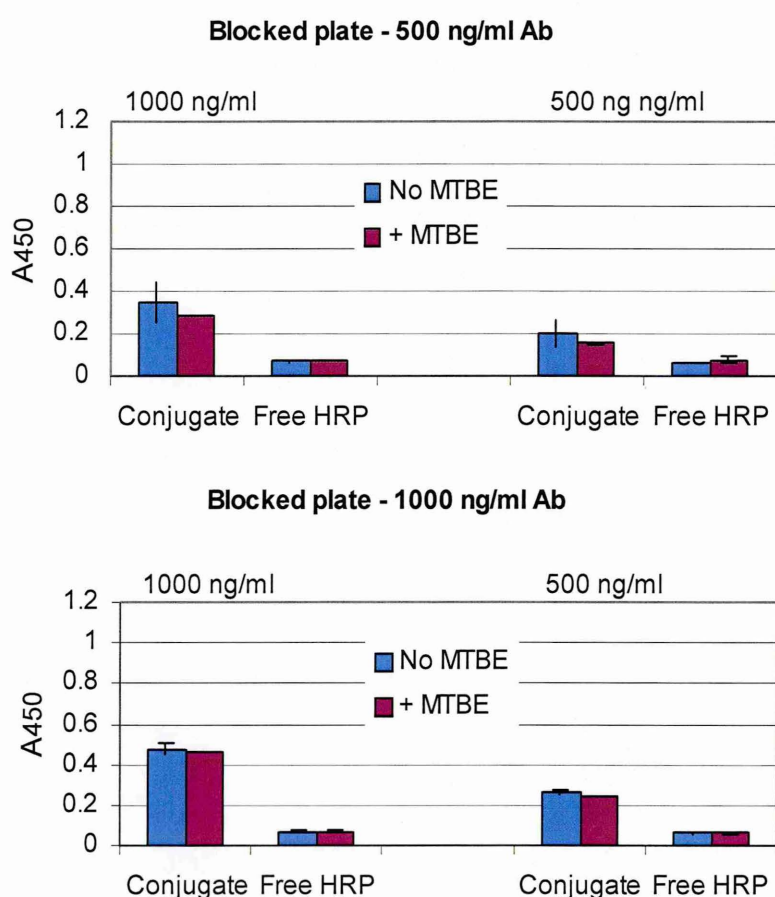


Fig. 6.50 Assay response with BSA-MODMO-HRP conjugates

Results show the assay responses from the BSA-MODMO-HRP conjugate on unblocked plates coated with two concentrations of primary antibody (see chart titles). The conjugate was tested at two concentrations (500 & 1000 ng/ml) on blocked plates (5.2.2.5). Samples were tested with & without MTBE (5000 ppm in PBS). Results are shown as mean values, $\pm$ SD, $n=4$.

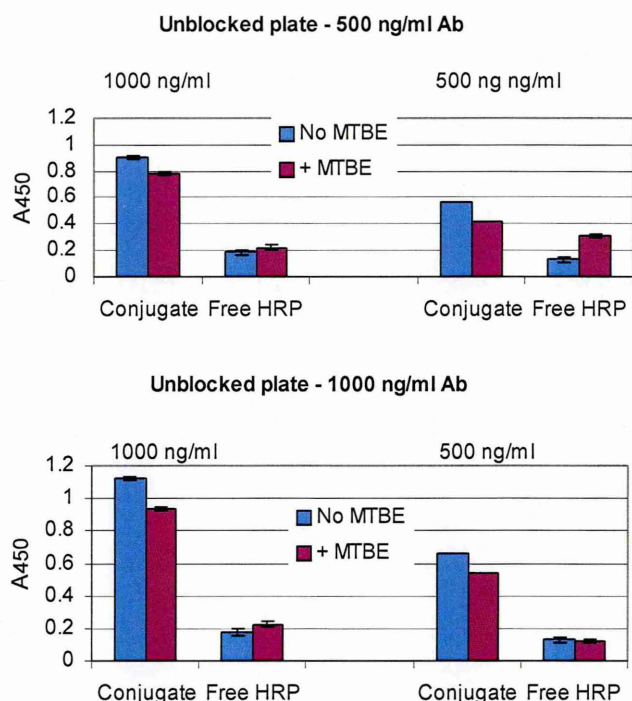


Fig. 6.51 Assay response with BSA-MODMO-HRP conjugates

Results show the assay responses from the BSA-MODMO-HRP conjugate on unblocked plates coated with two concentrations of primary antibody (see chart titles). Plates were left unblocked (5.2.2.5) and the conjugate was tested at two concentrations (500 & 1000 ng/ml). Samples were tested with and without MTBE (5000 ppm in PBS). Results are shown as mean values, $\pm$ SD, $n=4$.

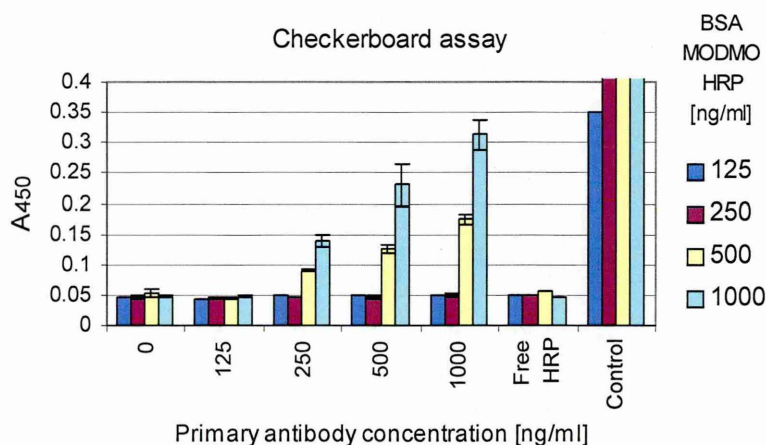


Fig. 6.52 Checkerboard assay using BSA-MODMO-HRP conjugate

A microtitre plate was coated with primary antibody (125-1000 ng/ml) and blocked with 1 % BSA (5.2.2.5.1). The BSA-MODMO-HRP conjugate was tested across these wells at four concentrations, as shown in the legend. Results are shown as mean values, $\pm$ SD, $n=4$. Controls ($n=2$) lacked PAb and blocking agent in order to determine the degree of NSB. The y-axis of the graph has been limited to 0.4 (A450) to show the detail between concentrations, the controls all exceeded 0.6 (control for 1000 ng/ml conjugate was 2.4).

The BSA-MODMO-HRP conjugate could distinguish between samples containing or not containing MTBE in both blocked and unblocked plates (Figs. 6.50-6.51). Assay signals were highest using PAb and BSA-MODMO-HRP conjugate at 1000 ng/ml, which was confirmed by the checkerboard assay (Fig. 6.52). These results are discussed further in Section 6.3.7.

6.3.5 Detection of MTBE by MODMO-HRP and BSA-MODMO-HRP conjugates

Both conjugate forms (MODMO-HRP and BSA-MODMO-HRP) were then tested with a range of MTBE solutions (Figs. 6.53-6.54) using both blocked and unblocked plates. Maximisation of the efficiency of immobilised PAb orientation was investigated in Section 6.3.6.

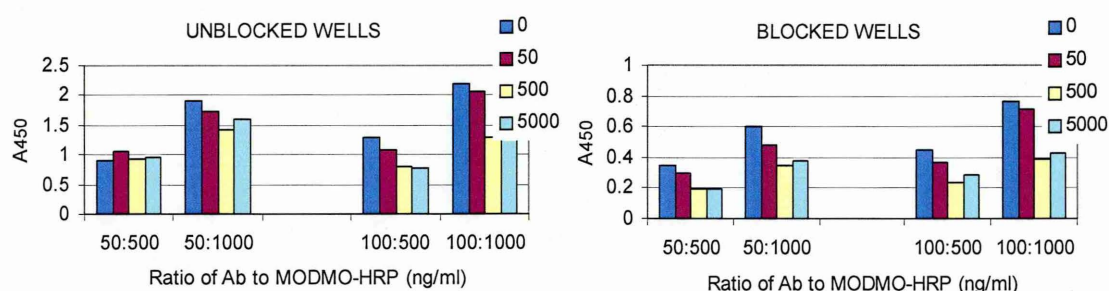


Fig. 6.53 Assay response with MODMO-HRP conjugates

Using conjugation Method 4 (5.2.2.2), MODMO-HRP conjugates (500 & 1000 ng/ml) and two PAb concentrations (500 & 1000 ng/ml) were tested with a range of MTBE solutions (5.2.2.6), as indicated in the legend key. Both blocked and unblocked plates were used. Results are shown as mean values, $\pm$ SD, $n=2$.

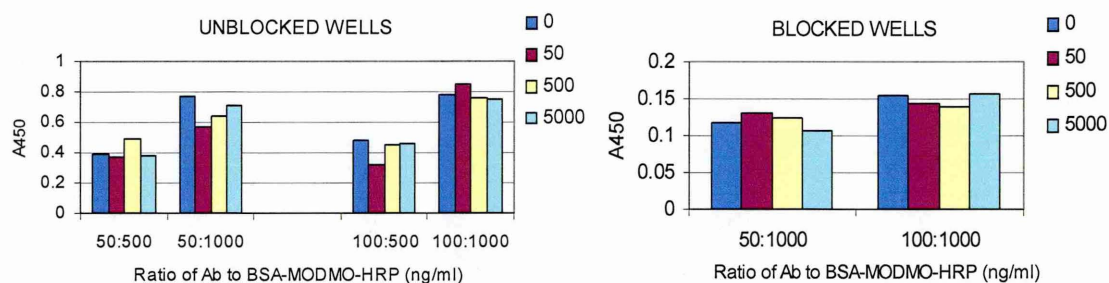


Fig. 6.54 Assay response with BSA-MODMO-HRP conjugates

BSA-MODMO-HRP conjugates (500 & 1000 ng/ml) and two PAb concentrations (500 & 1000 ng/ml) were tested with a range of MTBE solutions (5.2.2.6), as indicated in the legend key. Both blocked and unblocked plates were used. Results are shown as mean values, $\pm$ SD, $n=2$.

The range of MTBE concentrations (0, 50, 500, 500 ppm) could be distinguished using the MODMO-HRP in both blocked and unblocked wells. Highest signals (between 1 and 2 A450) were achieved in unblocked wells with a PAb coating of 100 ng/ml and MODMO-HRP concentration at 1000 ng/ml (Fig. 6.53). The BSA-MODMO-HRP conjugate failed to distinguish between any MTBE concentrations (Fig. 6.54).

6.3.6 Optimisation of primary antigen orientation

A final experiment attempted to maximise the efficiency of immobilised PAb orientation. As the binding of the PAb to the microtitre wells is most probably through random physical adsorption processes, it is possible that a proportion of the PAb molecules were not orientated for capture of the Ag. To avoid this, a plate, pre-coated with anti-IgG mouse Abs, was used to effectively bind the native PAb during plate preparation (5.2.2.7). This would bind the PAb via the homologous Fc fragment (crystallisable fragment, Chapter 1, Fig. 1.6) and orient the Fab fragments, containing the antibody binding sites in a uniform manner and in a position away from the well wall. Therefore, a greater proportion of the immobilised PAb should be available for maximising Ag capture. Control assays were also performed using standard Nunc plates, as described previously, which were coated with PAb/blocker (5.2.2.7, Fig. 6.55).

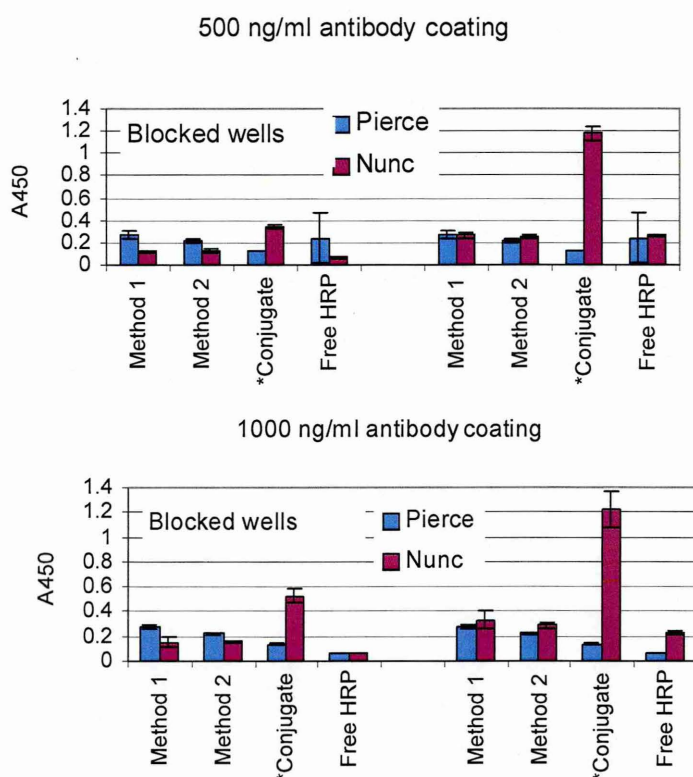


Fig. 6.55 Comparison of Ab-captured PAb and standard PAb coated plates

MODMO-HRP conjugate (prepared using Methods 1 & 2, 5.2.2.2) were tested and compared with BSA-MODMO-HRP conjugate (labelled *conjugate) in two plates. Each conjugate was tested at 1000 ng/ml. Standard Nunc plates were coated with PAb (5.2.2.3.1); Pierce plates were pre-coated with goat anti-PAb antibody (5.1.2.3). Free HRP was used as a control. Results are shown as mean values, SD $\pm$, n=4.

Assay signals were marginally improved by approximately 35% using the anti-PAb coated Pierce plates which had also been blocked with 1% BSA (w/v). Unblocked wells showed no improvement. Increasing the PAb coating concentration from 500 to 1000 ng/ml gave no discernible advantage. Results are discussed further in Section 6.3.7.

6.3.7 Conclusions and discussion

MODMO-HRP conjugations

Binding of a MODMO-HRP conjugate by a PAb raised against MODMO-BSA would require some degree of cross reactivity between the two Ags. Work with MODMO-HRP is consequently speculative in this respect. Assay responses were very low (maximum A450 ~0.12) using the MODMO-HRP conjugates produced following Methods 1 and 2 (5.2.2.2). However, in some assays, distinction could be seen (on blocked plates) between samples containing MTBE, suggesting some competition between free MTBE and the MODMO-HRP conjugate for the immobilised Ab binding sites (Fig. 6.47). This was more evident when using high concentrations (1000 ng/ml) of both immobilised PAb and the conjugate.

When tested on unblocked plates, assay responses increased from ~1.4 to 2.3 (A450). However, this was most probably a result of NSB, indicated by the high responses obtained with the free HRP controls (Fig. 6.48). This degree of NSB prevented any distinction between samples containing and not containing MTBE.

During the early conjugation process (Methods 1 and 2), it was possible that the HRP was denatured during the conjugation process, specifically when the MODMO-DMF solvent mixture was added. Proteins are known to be sensitive (i.e. denatured) to organic solvent concentrations $\geq 10\%$ v/v. It was also possible that the immobilised PAb was unable to recognise the hapten due to unfavourable changes in the conformation of the Ag-Ab binding sites. The PAb was raised against BSA-MODMO, but could recognise MODMO-HRP to a degree, most likely due to similar structural motifs present on the HRP molecule. The methods were subsequently revised (Methods 3 & 4, 5.2.2.2) and new conjugates were made. For these conjugations, the solvent was added slowly (and stirred) to prevent any sudden high increase in concentration. These new conjugates were tested by immunoassay, without any MTBE present, with the specific aim of improving the assay signal (in the absence of MTBE), which would be indicative of improved conjugate quality and consequently better recognition by the PAb (Fig. 6.49). Methods 3 & 4 did produce better conjugates, with a four-fold signal increase to ~0.4 (A450).

BSA-MODMO-HRP conjugates

Conjugation of the BSA-MODMO-HRP was a 2-step conjugation process, exploiting the particular chemical functionality of the BSA protein (rich in cysteine residues and charged amino acid side chains, lysine and arginine). Maxibind™ BSA carrier protein (5.1.2.2) was conjugated to MODMO following a standard protein to aldehyde-bearing hapten (MODMO)

protocol provided by the manufacturer (Uptima, 5.1.2.2). The Maxibind BSA protein had been modified with ethylene diamine to induce a cationised molecule, increasing the number of reactive amine groups by two-fold (approximately 70 participating functional groups as opposed to 35). Covalent binding of this cationised BSA to the MODMO was achieved by the classical Schiff base reaction, followed by reduction of the imine with cyanoborohydride (Fig. 6.56).

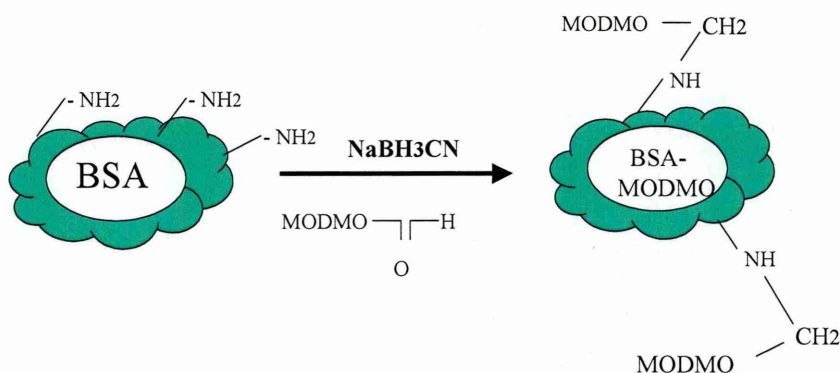


Fig. 6.56 Diagram of BSA-MODMO conjugation

The amine groups (-NH_2) present on the BSA were covalently linked to the aldehyde end of the MODMO through the formation of a Schiff base (Fig. 6.44), subsequently reduced by NaBH_3CN .

This procedure was used to provide BSA-MODMO Ag for use as the immunogen to raise the monoclonal PAb (by Diaclone SAS) and this Ag-conjugate (Ag) was also used throughout the ELISA development in this work (the Ag-conjugation procedure used is shown in Fig. 6.56). The BSA-MODMO-HRP conjugation tested in comparison was also supplied by Diaclone (5.2.2.4), reportedly using the same procedure (as Fig. 6.56) combined with a secondary coupling step, also a standard protocol supplied by Uptima. It is known that BSA contains a number of carboxylate groups (-COOH) which can bind to amine groups (-NH_2) present on HRP through formation of peptide bonds. Uptima provides a protocol for achieving this with the cationised BSA using a carbodiimide linking agent. It is possible that this was made in conjunction with the BSA-MODMO conjugation. However, Diaclone reported that a proprietary enzyme amplification step was employed and so the precise BSA-MODMO-HRP conjugation route taken remains proprietary to Diaclone. The resulting conjugate would have required precise purification, most probably through an HPLC column, in order to select the correct fraction, according to the predicted MW of the conjugate.

The BSA-MODMO-HRP protein complex was tested using a checkerboard assay to determine the optimal ratio of Ab to conjugate (Fig. 6.52). Due to the presence of BSA, which readily bind to the microtitre wells via physical adsorption under specified conditions, a secondary control, containing no PAb was used in unblocked wells. Highest binding occurred using both Ab and

the conjugate at 1000 ng/ml concentrations, with an absorbance of ~ 0.32 , which equated to that obtained in assays with MODMO-HRP. Further assays were conducted to determine the conjugate ability to compete for the Ab binding sites in the presence of free MTBE.

Detection of MTBE by both MODMO-HRP and BSA-MODMO-HRP conjugates

Fig. 6.53 shows that with the MODMO-HRP conjugates, and using either blocked or unblocked wells, the greatest assay sensitivity was between 50-500 ppm MTBE using an PAb coating of 100 ng/well (total PAb added) and MODMO-HRP (1 $\mu\text{g/ml}$). This reflects the limited binding of the PAb demonstrated in the early, competitive ELISAs.

The BSA-MODMO-HRP conjugates produced an even more insensitive ELISA (Fig. 6.54). When tested with a range of MTBE solutions (containing 0, 50, 500 & 5000 ppm), no distinction could be made between the assay signals. Therefore, a large proportion of the assay response was most probably a result of NSB to the well walls by the HRP and BSA proteins.

Optimisation of antibody orientation

It was possible that the low responses achieved by both conjugates (MODMO-HRP and BSA-MODMO-HRP) were due to poor orientation of PAb immobilised on the wells. To ensure improved antibody immobilisation, polyclonal anti-IgG pre-coated plates (5.1.2.3) were used to capture the PAb during the PAb-coating procedure. This would only bind to the Fc fragment of the PAb, such that the antibody binding sites were not involved in the immobilisation mechanism, leaving the bulk Fab antibody population (Chapter 1, Fig. 1.6) more amenable for later antigen capture during the immunoassay procedure.

MODMO-HRP assay signals increased by $\sim 35\%$ using PAb capture and optimised orientation in blocked wells (Fig. 6.55), with low NSB (absorbencies ~ 0.15), confirmed by the lower binding of free HRP. In contrast, the BSA-MODMO-HRP response (in unblocked plates) fell dramatically from 1.2 (standard Nunc plate) to 0.18 (A450) in the unblocked PAb capture plate, giving further validation to the suggestion that binding was non-specific.

Considering these results and taking into consideration the additional steps involved in preparing these conjugates, it was concluded that there was no significant benefit in continuing with these conjugations. The transposition of the ELISA into other formats (6.4-6.5) would therefore employ the same immunochemical reagents (BSA-MODMO Ag), PAb and 2Ab-HRP).

6.4 ENZYME-LINKED IMMUNO-FLOWTHROUGH ASSAY (ELIFA)

As described in Chapter 1 (Section 1.6.5), enzyme-linked flow through assays are more recent IA technologies developed primarily for testing water samples for contaminants. The ELIFA concept is very similar to that of the ELISA, where a primary antibody (PAb) captures the contaminant (Ag) and is detected by an enzymatic reaction with a substrate. An advantage of ELIFA is that the passage of solutions through a membrane can increase the rate of interactions between PAb and Ag, whereas in an ELISA, this is more diffusion limited. A consequence of this increased interaction efficiency is that assay times can be significantly reduced. ELIFA systems can also be transformed into hand-held portable ‘one-shot’ detection devices. An excellent example of such an ELIFA device is the Clear Blue pregnancy test (Unipath Ltd., Bedford, UK). Other examples are provided in Section 1.6.5.

6.4.1 Membrane selection

The first step for developing an ELIFA system was to find a membrane which would exhibit acceptable immobilisation chemistry with respect to the immuno-reagents (as used for the ELISA) and which would exhibit low NSB. Four membranes were chosen for their different binding properties (Section 1.6.5) and screened for NSB. Three different blocking agents were tested on each membrane for their capacity to prevent NSB by the second-HRP reagent - either s-HRP (200 ng/ml) or 2Ab-HRP (500 ng/ml) after 30 min. exposure in 4 ml solution (Fig. 6.57). Full methods are given in Chapter 5 (5.2.3.2).

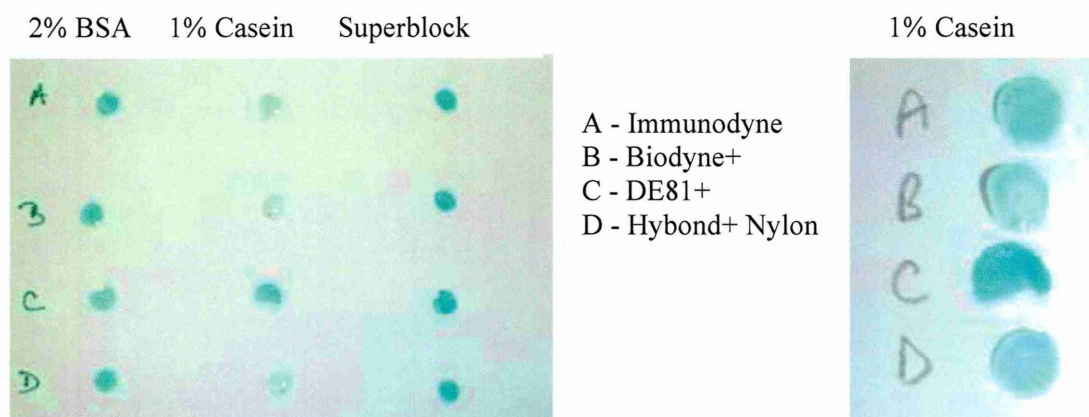


Fig. 6.57 Screening for NSB by 2Ab-HRP on 4 different membranes

Variation in NSB by the 2Ab-HRP on four different membranes (A-D) and the efficiency of 3 blocking buffers, as indicated. Superblock was used undiluted. Assays were conducted without PAb. Membranes were incubated in 4 ml of 2Ab-HRP for 30 min. Colour development was 10 min. (5.2.3.2.1). A close-up of the casein-blocked membranes is shown (r).

When the post-HRP washing step was extended for 30 min. in a repeat set of experiments, the DE81+ (DEAE-cellulose) membrane completely disintegrated. DE81+ gave high NSB with both HRP label combinations and all BBs and so was not tested further.

From these screening results, Casein proved the most effective BB for Immunodyne, Biodyne+ and Hybond+ membranes using the 2Ab-HRP label. Conversely, when the same membranes and blockers were tested using the s-HRP label, 2% (w/v) BSA was more effective against NSB on Immunodyne and Biodyne+ membranes, it was found to be even more effective when reduced to 1% w/v (Fig. 6.58).

A second series of experiments were conducted with Biodyne+, Hybond+ and Immunodyne membranes. In these assays, a direct comparison was made between assays with or without PAb (5.2.3.2.2). Results from the two positively-charged membranes Biodyne+ and Hybond+ are shown in Fig. 6.58.

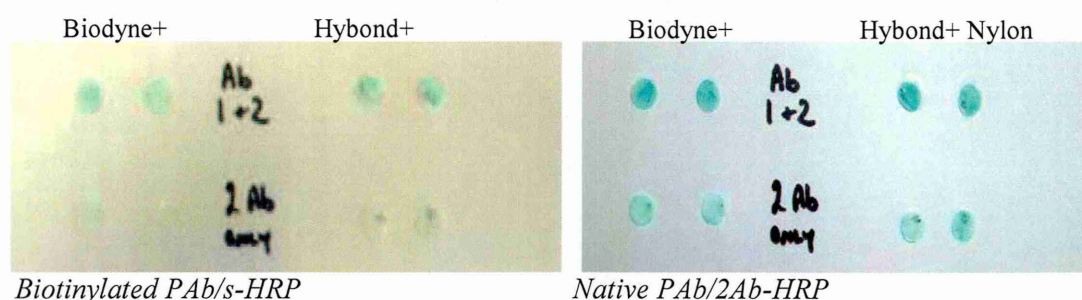


Fig. 6.58 Comparison of assay response with and without native PAb

Biodyne+ and Hybond+ membranes were tested in the presence of PAb (Ab 1+2), and absence of PAb (2 Ab). Assays using biotinylated PAb/s-HRP were blocked with 0.1% (w/v) BSA; assays with native PAb/2AB-HRP were blocked with 1% casein. Colour development was 10 min. (5.2.3.2.2).

BSA (1% w/v) and Superblock BB were most effective in limiting NSB from s-HRP on Immunodyne, Biodyne+ and Hybond+ membranes (Table 6.4). However, as found with the ELISA, native PAb showed overall better binding ability and subsequent detection by 2Ab-HRP through increased assay signals (visually assessed colour). With native PAb/2Ab-HRP, Casein and Superblock BB were most effective in limiting NSB. The addition of Tween 20 in the final PBS washing step appeared to aid removal of excess HRP-conjugate, reducing overall NSB.

6.4.2 Complete assays conducted on membrane surfaces in Petri dishes

The next step was to see if full assays could be conducted on the membrane surface and if free MTBE could be detected. If this was the case, then a colour loss should occur, as MTBE would block the PAb binding sites and prevent PAb binding to the immobilised Ag within the

membrane. In contrast to the previous experiments, the Ag coating step was by 1 hr incubation in an Ag solution, rather than through direct pipetting of Ag onto the membrane surface (5.2.3.3). All 3 blocking buffers (BBs) were tested with each PAb/HRP combination. Results using native PAb/2Ab-HRP-HRP with Immunodyne and Biodyne+ membranes are shown in Fig. 6.59.

Table 6.4 Different blocking agent efficiencies on ELISA membranes

Each membrane was tested with each BB either with or without PAb. Both biotinylated (b-PAb) and native PAb were then tested with their respective HRP labelling reagents (s-HRP or 2Ab-HRP respectively). Full assays were therefore conducted on each membrane. The most effective BB for each membrane and immuno-reagent combination are shown.

Membrane	Reagent combination	
	b-PAb & s-HRP	PAb & 2Ab-HRP
Biodyne+	BSA/SB	Any
Immunodyne	BSA	BSA/Casein
DE81+	NSB *	NSB *
Hybond+	BSA	Casein

* Non-specific binding (NSB) refers to the high NSB exhibited without PAb. Results were visually assessed and recorded after 10 min. colour development.

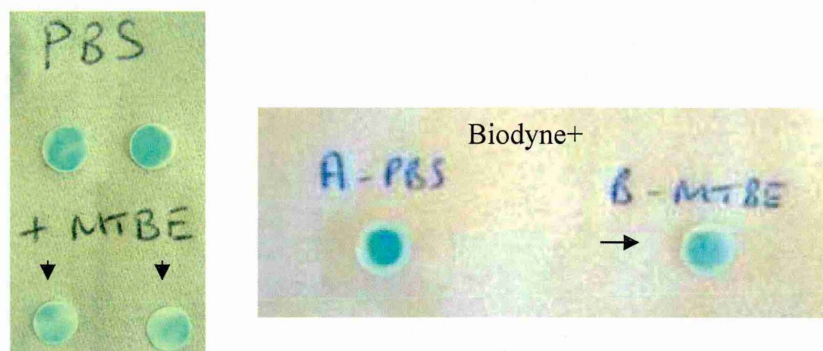


Fig. 6.59 Native PAb/2Ab-HRP assays on Immunodyne and Biodyne+ membranes

Assays were conducted with and without MTBE on Ag-coated membranes (5.2.3.3). Results show Immunodyne, blocked with 1% BSA (left) and Biodyne+, blocked with 1% casein (right). Colour development was 2 min. Assays with MTBE (5000 ppm) are arrowed.

The Ag bound sufficiently well when applied in solution to coat the membranes, rather than by direct application (by pipetting onto the membrane surface, as in 5.2.3.1). Detection of MTBE was achieved using Immunodyne and Biodyne+ membranes (Fig. 6.59), but results varied according to BB choices, and whether native or biotinylated PAb (and corresponding HRP-labels) were used. The Hybond+ Nylon membrane performed poorly at this stage, with little low colour produced by biotinylated PAb/s-HRP, regardless of the BB used. Whilst native PAb/2Ab-HRP produced higher colour, assays with MTBE present (5000 ppm) could not be differentiated from those without MTBE (PBS).

In order to evaluate the membranes more precisely, full ELIFAs were conducted in the flow cells, with each reagent pulled through the membrane, using the experimental arrangement described in Chapter 1. This stage of the work also required extensive, novel method development.

6.4.3 Evaluation of membranes using ELIFA Protocol 1

Protocol 1 was devised using the same volumes and concentrations of reagent solutions as used for the ELISA development (5.2.1.6). Membranes were tested using both biotinylated PAB/s-HRP and native PAB/2Ab-HRP combinations (5.2.3.4). The Hybond+ Nylon membranes showed poor PAB/Ag binding, indicated by the lack of membrane colouration and poor distinction between PBS and PBS/MTBE (Fig. 6.60).

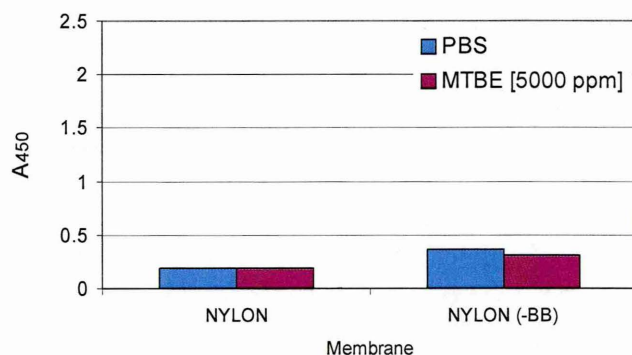


Fig. 6.60 ELIFA assay signals from Hybond+ Nylon and DE81 membranes

Hybond+ Nylon membrane, blocked with 1% BSA (w/v) were tested by ELIFA following Protocol 1 (5.2.3.4). PAB/2Ab-HRP results are shown. Unblocked Hybond+ Nylon membrane is indicated as [-BB]. MTBE (5000 ppm) was prepared in PBS.

Immunodyne and Biodyne membranes could distinguish between the absence and presence of MTBE (5000 ppm in PBS) using either PAB/HRP combination to a degree, although signal differences were small (0.2 A450) and variable. In view of these results, further developmental work concentrated on the Immunodyne and Biodyne+ membranes.

As the Immunodyne membrane had previously showed largest responses using the biotinylated PAB/s-HRP combination, these reagents were retained throughout the development of a working protocol using this membrane (Protocols 2-5, 5.2.3.5). Conversely, the Biodyne+ membrane appeared to respond with both PAB/HRP combinations and so, after extensive revisions, a further ELIFA protocol was developed for Biodyne+ (Protocol 6, 5.2.3.6.1). Results are presented for the Immunodyne-based ELIFA and then the Biodyne+ ELIFA.

6.4.4 Evaluation of Immunodyne membrane and further optimisations

Protocols 2-4 tested the ELIFA using pre-Ag coated Immunodyne membrane, which was blocked with BSA after insertion in the flow cell (5.2.3.5). In Protocol 5, the Ag was immobilised by pumping through the membrane after insertion within the flow-cell. As the biotinylated PAb bound strongly to the immobilised Ag, free MTBE was incubated with the PAb prior to running the ELIFA in Protocol 5. Full modifications are provided in 5.2.3.5.

Fig. 6.61 shows the gradating improvement of the ELIFA Protocol (2-4), with clearest differentiation between blank samples and those containing MTBE (5000 ppm) obtained using Protocol 4. However, this was again improved using Protocol 5, where PAb was incubated with free MTBE prior to running the ELIFA. This Protocol also used a constant slow flow rate (0.130 ml/min) throughout the ELIFA (5.2.3.5.1).

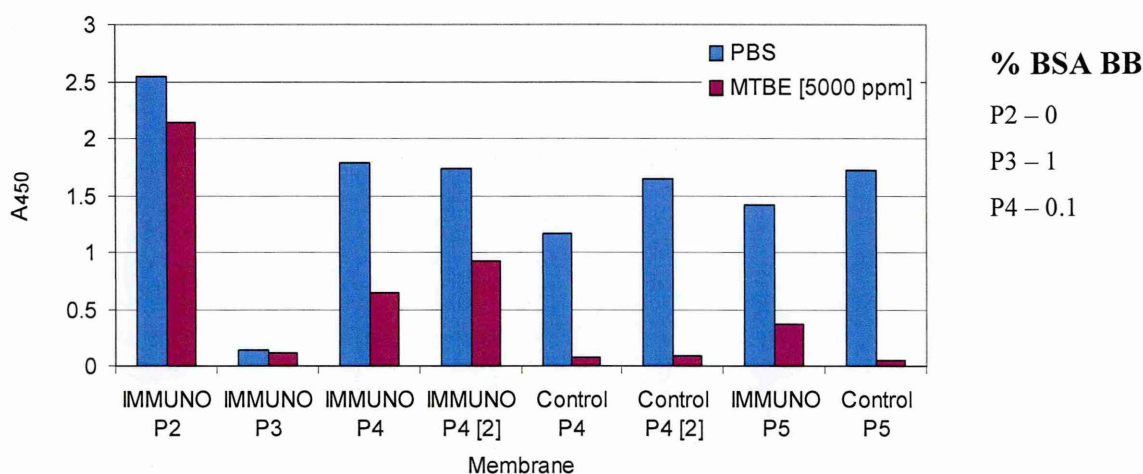


Fig. 6.61 Evaluation of Immunodyne membrane for ELIFA

P2-P5 relates to Protocols 2-5 respectively using biotinylated PAb/s-HRP (5.2.3.5) and BSA BB (indicated right). Repeat P4 ELIFAs are marked P4 [2]. PBS assays lacked MTBE. Controls were ELISAs run simultaneously with the P4 and P5 ELIFAs, mean results are shown $\pm$ SD, $n=3$.

6.4.5 Development of the Biodyne+ ELIFA

ELIFA Protocol 5 (developed using Immunodyne membrane) was then applied to both Immunodyne and Biodyne+ membranes and the results compared (Fig. 6.62). Both membranes clearly differentiated between samples with or without MTBE (5000 ppm), although the maximum signal for the Biodyne+ was marginally lower.

The Protocol was again modified to optimise the ELIFA specifically for Biodyne+ membrane. A revised pump speed (0.05 ml/min.) was used for Ag and PAb/s-HRP reagents (Protocol 6, 5.2.3.6.1). Previous results had indicated that BSA (1% w/v) and SB (undiluted) were the most

suitable BBs for this membrane when using biotinylated PAb/s-HRP (Table 6.4). Fig. 6.63 shows the ELIFA results obtained using these two BBs. Surprisingly, both BSA and Superblock failed to prevent NSB by the 2Ab-HRP, resulting in no detection of free MTBE. Protocol 6 was then applied using the native PAb/2Ab-HRP combination (Fig. 6.64), where repeated ELIFAs were conducted using the Superblock BB.

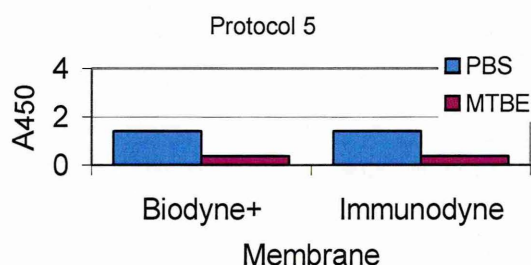


Fig. 6.62 Preliminary evaluation of Biodyne+ membrane for ELIFA

Biodyne+ membrane was tested following ELIFA Protocol 5 (5.2.3.5.1) and is compared with Immunodyne. Biotinylated PAb/s-HRP with 0.1% (w/v) BSA BB were used for both ELIFAs.

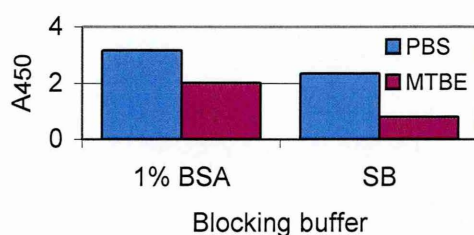


Fig. 6.63 Biodyne+ ELIFA results (Protocol 6) with two different BBs

ELIFAs were conducted with Biodyne+ membrane blocked with either 1% BSA (w/v) or Superblock (SB), following Protocol 6. Biotinylated PAb and s-HRP were used for both ELIFAs (5.2.3.6.1).

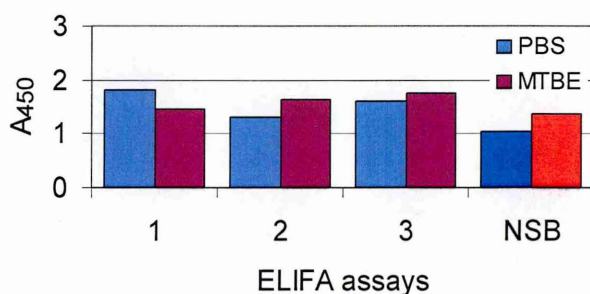


Fig. 6.64 NSB from 2Ab-HRP on Biodyne+ membranes blocked with SB

When the ELIFA employed native PAb and 2Ab-HRP (5.2.3.6.2), a large increase in NSB (from the 2Ab-HRP) prevented any detection of MTBE. Results show membranes blocked with undiluted Superblock (SB) and are from 3 separate, repeat ELIFAs. NSB controls lacked either Ag (shown in blue) or PAb (red). ELIFAs otherwise followed Protocol 6 (5.2.3.6.1).

Both buffers (1% BSA and Superblock) were replaced with Casein (in TBS) at two different concentrations (1% & 0.1% v/v). Early screening experiments had shown Casein to be the most effective BB using Biodyne+ membrane and the PAB/2Ab-HRP combination (6.4.1). ELIFAs were then run with the native PAB/2Ab-HRP combination, following Protocol 6 (5.2.3.6.2). Fig. 6.65 shows that with 1% Casein, there was little improvement. However, 0.1% Casein was much more efficient at reducing the NSB and showed a clear distinction between samples with/without MTBE (5000 ppm in PBS). Using this combination of membrane/reagents and the revised Biodyne+ Protocol (Protocol 6), the ELIFA performance was better than those achieved with the Immunodyne ELIFA (Fig. 6.61).

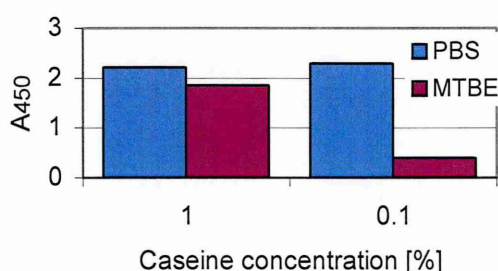


Fig. 6.65 ELIFA performance using Casein BB with native PAB

ELIFAs were conducted on Biodyne+ membrane, blocked with either of two concentrations of Casein (1 and 0.1% v/v, 5.2.3.6.2). Assays were run using native PAB/2Ab-HRP.

6.4.6 Conclusions and discussion

Assays for MTBE conducted on membrane discs incubated in solutions contained in Petri dishes showed poor repeatability and a relatively high degree of NSB (Fig. 6.59). This occurred whether membranes were pre-Ag coated (~50 ng/disc) by pipetting and drying; or coated by incubating the membranes with Ag in solution. This is perhaps not unexpected because introducing protein solution onto highly absorptive microporous membrane matrices by such non-quantifiable methods, such as manual pipetting/gross immersion in bulk solutions is almost certain to introduce variations in the dynamics of the migration of any protein into and throughout each individual membrane disc. This will clearly then have consequences for variability of the invading protein's binding, either to the membrane itself or to other proteins already immobilised, possibly leading to significant disc-to-disc differences in both protein loading and distribution. Variability due to factors such as these is also likely to be exacerbated by the presence of an excess of (non-immobilised) sequential protein loading. However, these effects were minimised in the full ELIFAs (using flow cells) as solutions were actively pulled through the membrane in a more controlled and consequently more reproducible way.

The choice of BB for preventing NSB differed radically between membranes and would again alter when changing the choice of reagents, between either biotinylated PAb/s-HRP or the native Pab/2Ab-HRP combination. This was observed both during the Petri dish experiments and later flow-cell ELIFAs. The drastic increase in NSB by the 2Ab-HRP can possibly be explained by the fact that the 2Ab-HRP conjugate is a much larger molecule than s-HRP, with many more potential binding sites. This conjugate therefore may bind non-specifically to the membranes (which are designed to bind proteins) and/or bind to the immobilised blocking proteins within the membrane structure. Alternatively, the membrane may well have become overloaded with proteins, forming a generalised congested barrier so that washing steps were less effective. The increase in NSB with higher concentrations of either BB (>1% w/v) seems to support this. Indeed, the best differentiation of samples with and without MTBE was achieved using 0.1% (w/v) BB concentrations.

Similarly, other variations during the development work (not illustrated) involved increasing both Ag (>250 μ l) and BB volumes (>800 μ l). Again, whilst much higher signals were obtained overall, there was no differentiation between samples containing MTBE and those which lacked MTBE, again suggesting protein overloading the support membrane, consequently blocking a large number of pore channels.

ELISA Protocol development

During the ELIFA developmental work, It was possible that altering the flow rates during the ELIFA may have caused flow change and hence shear effects that may possibly damage the fragile, immobilised PAb/Ag structures. The formation of air pockets within the flow cells and tubing can be problematic should pumping be inconsistent, or reagents pass too rapidly through the membrane. A constant flow rate (0.130 ml/min.) was used in Protocol 5. Whilst this improved the ELIFA with the Immunodyne membrane (Fig. 6.61), the Biodyne+ ELIFAs were more effective with a slower Ag/PAb/HRP-label flow rate (Protocol 6, 5.2.3.6.1). ELIFA data suggested that the method performed more effectively at slower flow rates, however, the development of a workable assay relies on balancing bind efficiencies with assay rapidity.

It was also found that if the PAb/MTBE was not mixed prior to ELIFA, there was no differentiation between samples with or without MTBE (5000 ppm). Thus, pre-incubation effectively gives the MTBE a PAb-binding advantage. The free MTBE blocks part of the PAb-binding region for the Ag, so that when the PAb/MTBE is then exposed to the Ag, binding is reduced. In the absence of MTBE, binding of the PAb to its Ag is likely to be of a higher affinity and hence less susceptible to disruption by subsequent addition of MTBE than to the competitive binding reduction seen when all three reagents are introduced simultaneously. A

PAb/MTBE pre-incubation favours the establishment of an PAb-MTBE binding equilibrium before its exposure to the Ag. Therefore, this PAb/sample incubation step is crucial.

The Immunodyne membrane had the practical disadvantage of having an active surface chemistry, which required storage under nitrogen, careful handling and the requirement to be used within 30 min. of air exposure. A new membrane, UltraBind[®] has since become available from Pall (Perbio Science UK Ltd., Tattenhall, UK), designed for the same applications as Immunodyne. This is a modified polyethersulfone membrane, with a high density of aldehyde functional groups, which provide effective covalent binding to amine groups on proteins. The surface chemistry is specifically designed to have extremely low, non-specific binding. The membrane's active chemistry is inert to moisture and heat, and requires no special storage. This may prove much more suitable for future MTBE ELIFAs of this type.

In conclusion, this work has demonstrated the proof of principle for transposing the MTBE ELISA into an ELIFA format. MTBE detection was possible using both Biodyne+ and Immunodyne membranes, but relied heavily on the choice of immuno-reagents and BB used. However, detection was only possible at relatively high MTBE concentrations (5000 ppm) and the lower limit of sensitivity was not determined.

It is envisaged that, should further work refine and improve the assay sensitivity, the current ELIFA experimental arrangement using flow cells could be miniaturised for field tests, such that the flow-through device would be a single-use, self-contained, hand-held plastic device. It could be manufactured by injection-moulding and ultrasonic welding, possible analogous in structure to the familiar 'Acrodisc' disposable filter units, made by Pall (Fig. 6.66). Water samples and assay reagents could be injected consecutively through the device, very much as small water samples are passed through a micro-filter. This could be aided with the use of a small, portable pump.



Fig. 6.66 Acrodisc disposable filter unit

This filter unit contains an internal, central filter disc. Samples are injected manually through the upper cannula and fractions collected from the underside.

If the MTBE ELIFA detection limits could be lowered sufficiently, a simple, portable visual pass/fail test device might be realised. A more sophisticated device could carry recesses for plugging in an emitter and detector, linked by fibre-optic cable to a portable reflectometer, in order to read colour intensity directly from the membrane surface (Fig. 6.67).

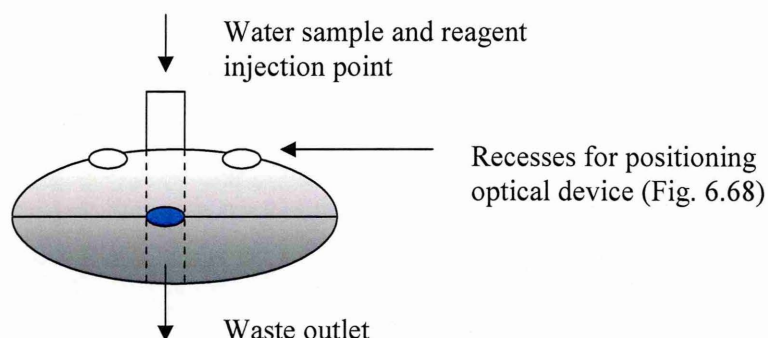


Fig. 6.67 Conceptual prototype, disposable hand-held ELIFA device

The proposed device consists of two plastic components, with an internal flow-through channel. A membrane is placed centrally. Reagents can be injected into the upper chamber and passed through the membrane in the desired sequence. Alternatively, solutions can be pulled through by means of a small, portable downstream pump. Final colour can be assessed on the membrane reflectometrically (as shown) or after removal and interrogation of the reaction product.

Whilst the final reactant (TMB) could be extracted from the device, either by syringe or by pumping throw and collecting the colour fraction and transferred to a vessel for determining the absorbance, an alternative method of colour detection could be advantageous.

During the development of this ELIFA, a commercially available 'Color Sensor Module (TCS230)' was obtained from Texas Advance Optoelectronic Solutions (TAOS, Texas, USA) and evaluated for potential use with the ELIFA (Fig. 6.68). The unit was supplied with a Parallax board, pre-loaded with a BASIC Stamp Editor programme for data analysis (Parallax Inc., California, USA). This was connected via a serial cable connector to a PC for data display.

The module powers two white external LEDs, either side of a 5.3 mm lens, which is positioned 25 mm from the board surface. The LEDs and lens are an optimal fixed distance from the surface by the 2.5 cm aluminium stand-offs, which can be modified if necessary. The lens detects reflected light over a 4 mm area, which is approximately the same area where colour development occurs on the membrane surface within the ELIFA flow-cells.

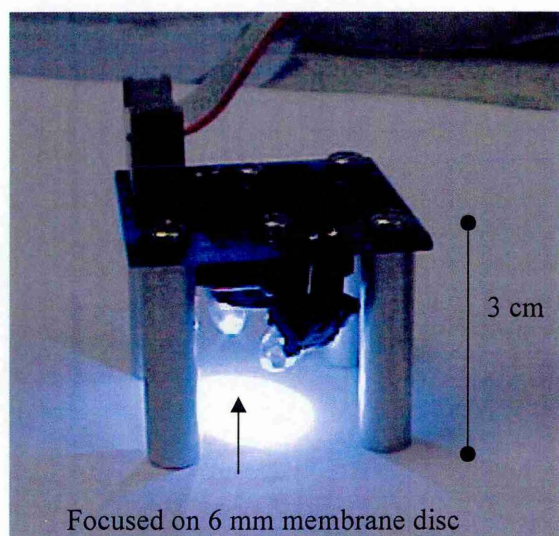


Fig. 6.68 The Colour Sensor Module evaluated for ELIFA

The TCS230 Colour Sensor Module (Taos, Texas, UK). The 2 LEDs are focused on the centre of a 6 mm membrane disc. Distance from the lens is fixed by the 2.5 cm aluminium standoffs. Reflected light is detected by the central, on-board lens (not visible).

Internally, the TCS230 has an array of photo-detectors, each with either a red, green, or blue (RGB) filter. An internal oscillator produces a square-wave output, whose frequency is proportional to the intensity of the chosen colour (~610, 550, 460 nm respectively). Any colour variation within the 4 mm² sensing area will be averaged by the software. Once calibrated, the on-board sensors produce an output voltage proportional to reflected light detected by the on-board lens (very similar to the reflectometric sensor principle, reported in Ch.2). A 3-channel A/D sends this information to a micro-controller, which then accesses programmed colour tables and converts the data into RGB co-ordinates. These values are then relayed and displayed on the PC programme. The unit is designed to be battery-powered, as LEDs require no warm-up time and take very little current. This device could potentially be adapted for use with the ELIFA, either in the current flow-cell format, or possibly with a miniaturised device, as suggested in Fig. 6.67.

This concluded the developmental work for the ELIFA. An alternative method of transposing the MTBE ELISA to an alternative format was by the immobilisation of the ELISA reagents onto magnetic beads, in place of the membrane (ELIFA) or microtitre well (ELISA) and this is reported in the following section (6.5).

6.5 DEVELOPMENT OF A MAGNETIC BEAD BASED ASSAY

The magnetic beads assay essentially follows the same methodology as ELISAs, where Ag is immobilised, in this instance onto magnetic beads. PAb is then added with a MTBE-contaminated water sample and this competes against the immobilised Ag for PAb binding sites. Detection is through secondary PAb capture by a polyclonal anti-PAb antibody (2Ab-HRP) labelled with HRP enzyme (Chapter 1, Fig. 1.6). It should be noted that only 6 assays were possible per test due to limitations imposed by the 6-tube capacity magnetic rack.

6.5.1 Development of the magnetic bead MTBE assay - Protocol 1

Protocol 1 was devised by adapting the basic ELISA procedure (developed in 5.2) and with regard to magnetic bead (MBs) concentrations and protein loading. Higher concentrations of both PAb and 2Ab-HRP were found to be necessary when compared to the ELISA method. NSB had previously shown to be primarily from the 2Ab-HRP and so this was firstly investigated (Fig. 6.66). Dynabeads were coated with Ag (5.2.4) and assays were performed using a preliminary Protocol (Protocol 1, 5.2.4.4). For determination of NSB (Fig. 6.69) by the 2Ab-HRP, no PAb was used (5.2.4.4.1). Assays were then conducted to determine the optimal PAb concentration and the optimal volume required per assay (with a total assay volume of 1 ml, Fig. 6.70).

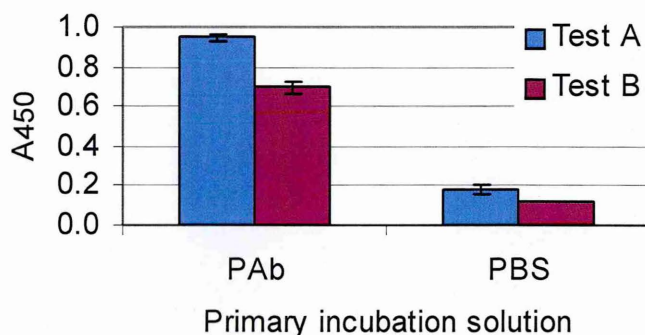


Fig. 6.69 NSB of 2Ab-HRP on magnetic beads

Two separate tests (A & B) were conducted to determine the degree of NSB by the 2Ab-HRP (5.2.4.4.1). Full assays were performed following Protocol 1 (5.2.4.3), labelled PAb in the figure. For determining NSB, in a second series of assays, the PAb was omitted in the primary incubation, shown as PBS in the figure. The CV between results ($n=6$) was $\pm 19\%$.

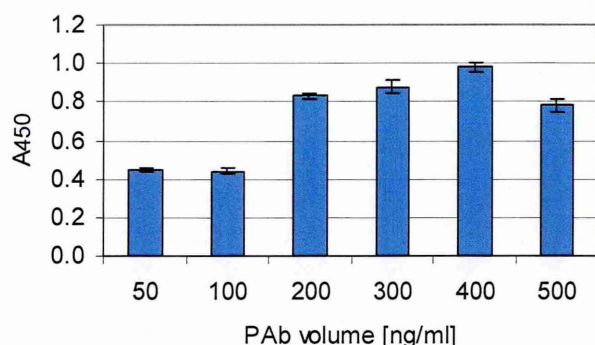


Fig. 6.70 Effect of increasing PAb concentration on magnetic bead assays

In each 1 ml assay, the concentration of PAb was increased (as indicated). A corresponding decrease in buffer A was made to maintain a total 1 ml assay volume (5.2.4.4.2). Assays were otherwise conducted as Protocol 1 (5.2.4.3). Results are shown $\pm$ SD, $n=4$. The CV was $\pm 5\%$.

6.5.1.1 Protocol 1: a modification

As results from Fig. 6.70 showed an increased response with increased amounts of PAb in the 1 ml test solution, Protocol 1 was amended. The amount of PAb added to the test solution was increased from 100 to 300 ng/ml. Correspondingly, the amount of buffer A was reduced to 200 μ l in order to keep the 1 ml assay volume (which also contained 400 μ l 'sample' and 100 μ l of Ag-coated beads).

The next step was to test the magnetic bead assay with known concentrations of MTBE using the amended Protocol 1). The repeatability of the assays was then examined (Fig. 6.71) and the stability of the Ag-coated T-beads was also investigated by testing both aged and freshly coated beads (aged Ag-coated beads had been stored at 4 $^{\circ}$ C for 7 days).

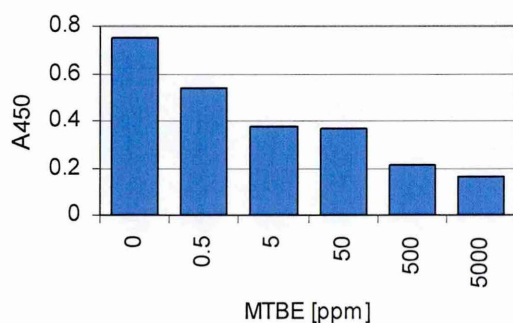


Fig. 6.71 Detection of MTBE with MB assay (Protocol 1)

A range of MTBE concentrations (in PBS) were tested using the competitive immunoassay format, using a PAb magnetic bead loading of 300 ng/ml. Assays were otherwise conducted as Protocol 1 (5.2.4.3).

6.5.1.2 Detection of MTBE by the MB assay and assay repeatability

The magnetic bead assay could detect MTBE down to 0.5 ppm using the amended Protocol 1 (5.2.4.5). Repeatability of the assay was also examined and the CV between replicate assays was 8% (Fig. 6.72).

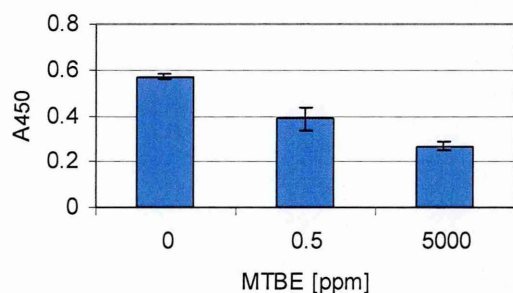


Fig. 6.72 Repeatability of magnetic bead assays

Three spiked solutions of MTBE were repeatedly tested using the amended Protocol 1 (5.2.4.6.2). Results are shown $\pm$ SD, $n=6$. The CV between assays was $\pm 8\%$, except for assays using the 0.5 ppm MTBE, which was $\pm 13\%$.

6.5.2 Ag coating protocol revision

In order to reduce waste of reagents, the ratio of the Ag:beads was recalculated and the Ag-coating method subsequently adapted to give a final Ag:bead coating concentration of $1 \mu\text{g}: 2 \times 10^8$ beads per 1 ml assay (5.2.4.7). To examine the effect of the different Ag-coating concentrations, three sets of MBs were coated with Ag at 0.5, 0.5 and $1.0 \mu\text{g}/\text{ml}$ (all with 2×10^8 beads in a total volume of 1 ml (5.2.4.8). Each set of beads was then tested with three MTBE concentrations. Results showed that optimum Ag-coating concentration was $1 \mu\text{g}: 2 \times 10^8$ beads (Fig. 6.73).

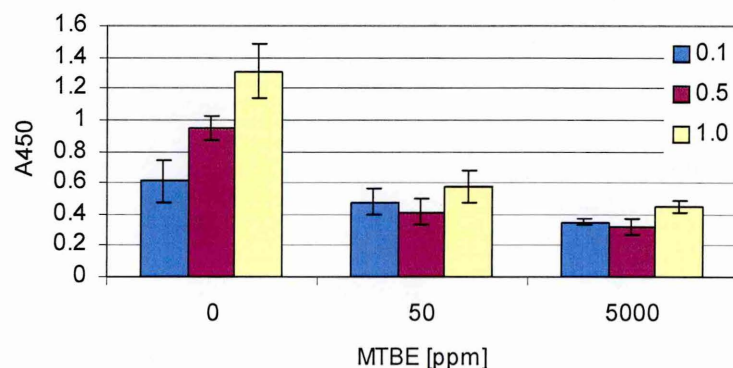


Fig. 6.73 Effect of reducing Ag-coating concentration

Three sets of beads were coated with Ag concentrations of 0.1, 0.5 & $1.0 \mu\text{g}$ per 2×10^8 beads in 1 ml assays (5.2.4.8). Each set of beads was tested with three MTBE concentrations, as shown. TMB colour substrate and Stop solution volumes were also each reduced from 500 to 250 μl . Results are shown as mean values, $\pm$ SD, $n=2$. CVs were less than 14%.

6.5.3 Protocol 1: further modifications

Ag-coating concentration: Results from Fig. 6.73 confirmed that the optimal 1 µg Ag: 2×10^8 beads Ag-coating ratio gave improved results when compared to the previous coating procedure (5.2.4.2), which gave assay signals below 1.0 (A₄₅₀), regardless of PAb concentration. Any further reduction of Ag: 2×10^8 beads also resulted in reduced assays signals below 1.0 (A₄₅₀). This revision was subsequently retained for future bead preparations.

Colour substrate: In addition, using a reduced volume (250 µl) of TMB, together with continuous mixing (10 min.) during the colour development stage gave improved assay signals (A₄₅₀) from ~0.6 (Fig. 6.72) to 1.4 (Fig. 6.73). This amendment to Protocol 1 was consequently retained for future assays.

6.5.3.1 Effect of increased PAb incubation times

Further assays were conducted incorporating the modifications to Protocol 1. A comparison of assays using PAb at 100 ng/ml and the optimal 300 ng/ml concentration is show in Fig. 6.74. In order to try and increase assay signals further (>1.0), the PAb incubation was increased from 30 min. to 60 min.

6.5.4 Magnetic bead assay - Protocol 2

Protocol 2 incorporated the modifications from Protocol 1 obtained from the early development work described previously (6.5.1-6.5.3). Protocol 2 was then used for the following investigations, using beads prepared using the revised coating calculations. Full details of Protocol 2 is provided in Chapter 5 (5.2.4.1.1).

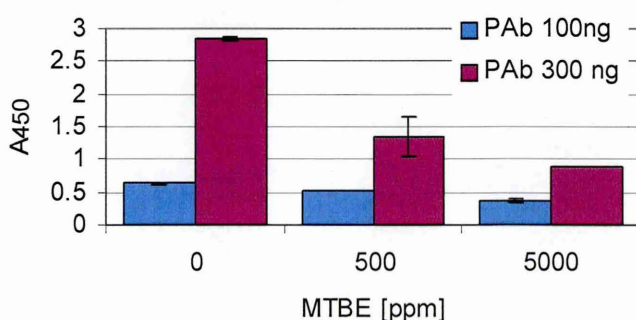


Fig. 6.74 Effect of increased PAb incubation

Magnetic bead assays were incubated for 60 min. (as opposed to 30 min.) with PAb at either 100 or 300 ng per 1 ml assay. Tests were performed in duplicate, n=2.

Further assays were conducted following Protocol 2 (5.2.4.11) using a range of MTBE concentrations (0, 50 & 5000 ppm in PBS). The magnetic beads were freshly coated with 2 concentrations of Ag (1.0 or 10 $\mu\text{g/ml}$ per 2×10^8 beads). In a second series of assays, Tween 20 detergent (T20, 0.2% v/v) was added to wash buffer C (referred to as buffer C/T), which was used for all washing steps in Protocol 2. Both sets of results are shown in Fig. 6.75.

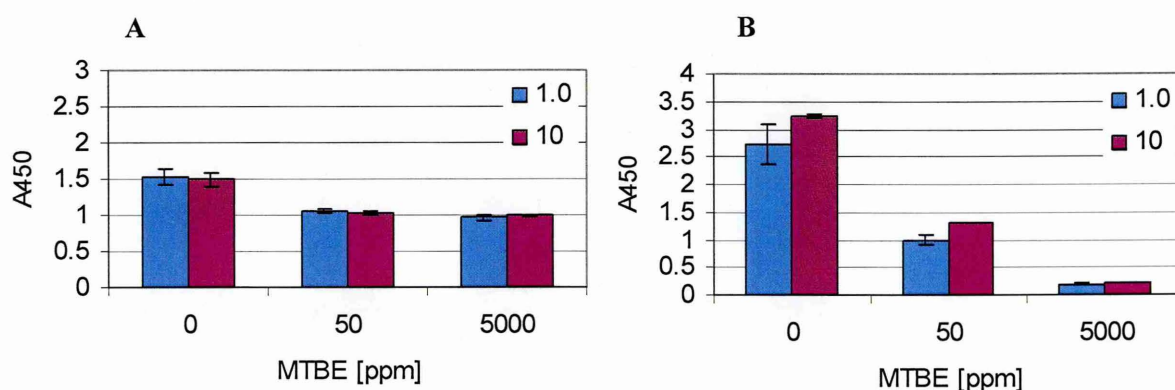


Fig. 6.75 Effects of T20 in wash steps

Beads were coated with 2 concentrations of Ag (1.0 or 10 μg Ag: 2×10^8 beads). (A) A range of MTBE concentrations were tested using the Protocol 2 (5.2.4.11), $n=2$. (B) Assays were then repeated but with extended wash steps using wash buffer C/T, containing 0.2% v/v T20, $n=2$.

6.5.4.1 Protocol 2: a final modification

Due to the effectiveness of the revised washing buffer C/T (containing 0.2% v/v T20), this condition was retained in Protocol 2.

6.5.5 Detection of MTBE and MODMO by magnetic bead assay

A extended range of MTBE and MODMO concentrations were then tested by the magnetic bead MTBE assay following Protocol 2 and compared (Fig. 6.76). Similarly, MODMO detection by the bead assay was compared to its detection by the ELISA Protocol 3 (Fig. 6.77), which is described in Chapter 5 (5.2.1.6).

Fig. 6.76 shows that detection of MTBE was achievable between 0.5-500 ppm using the magnetic bead assay, which was comparable to that obtained using the optimised ELISA (6.2.3). Similarly, as also found with the ELISA, the assay was much more sensitive to MODMO, which was what the PAb was raised against. Linear detection of MODMO can be seen between 0.05-5 ppb, as opposed to ppm for MTBE (Fig. 6.76). A larger range of MODMO dilutions were made (from 10,000 ppm down to 0.005 ppb) and simultaneously tested using both the magnetic bead assay (Protocol 2, 5.2.4.11) and by ELISA (5.2.1.6). Fig. 6.77 shows the results from each method, which both indicated a MODMO IC₅₀ of 50 ppb.

These results were then combined to examine the variation between each method (Fig. 6.78), where the IC50 again was confirmed as 50 ppb. The CVs for each were less than 0.42%.

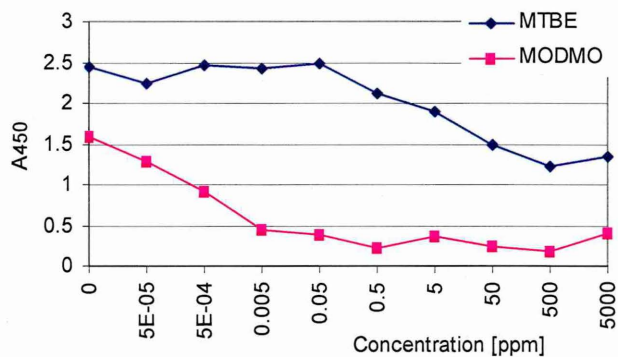


Fig. 6.76 Detection of MTBE and MODMO by magnetic bead assay
MTBE and MODMO concentrations were tested following Protocol 2 (5.2.4.11), incorporating the wash buffer modification (T20) in 6.5.4.1.

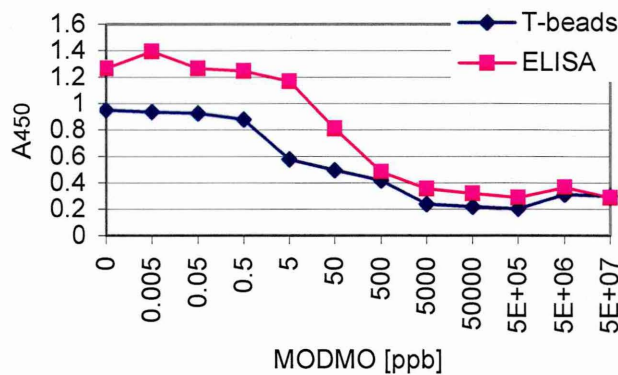


Fig. 6.77 Comparison of MODMO detection by magnetic bead assay and ELISA
Low range (ppb) concentrations of MODMO were tested by T-beads and ELISA (5.2.1.6). Magnetic bead assays were following the Protocol 2 (5.2.4.11), incorporating the wash buffer modification (T20) in 6.5.4.1. ELISA results are shown as mean values, n=8.

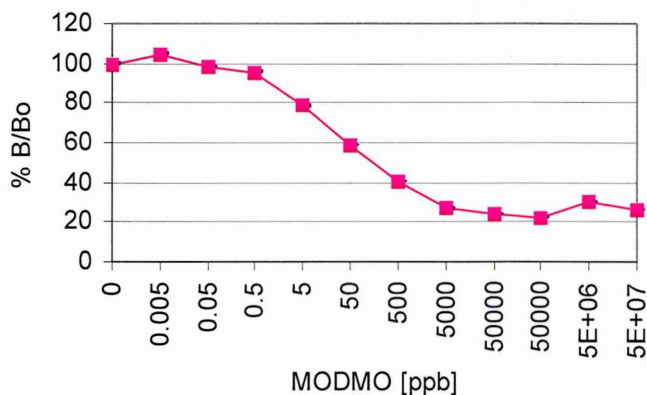


Fig. 6.78 Combined comparison of MODMO detection by magnetic bead assay and ELISA
The figure shows the combined results from both magnetic bead and ELISA assays from Fig. 6.77, expressed as % B/Bo. The CVs for each point were less than 0.42%.

6.6 CONCLUSIONS AND DISCUSSION

The transposition of the MTBE ELISA onto magnetic beads (MBs) was in itself quite a novel step, as very little information is available regarding bead- based immunoassays, as the beads have generally been used as a protein capture/separation technique, rather than for a competitive immunoassay (Chapter 1, Section 1.6.6). Therefore, it was a logical first step to transpose the optimised MTBE ELISA to the magnetic beads using the same reagent concentrations for initial comparison. The MB assay was then further optimised through a series of investigations.

Ag-magnetic bead coating procedure and stability of Ag-coated beads

It was found that the amount of Ag required for bead coating could be significantly reduced from the first coating procedure (48 μg Ag: 1.48×10^9 beads). The revised Ag-coating procedure (1 μg Ag: 2×10^8 beads) resulted in both reduced Ag and bead concentrations (50 and 7-fold respectively), yet provided more beads physically available for each Ag molecule. No significant differences were found between assays using freshly coated beads and beads that had been coated and stored for 7 days, suggesting acceptable stability once coated and stored at 4 °C.

Immunoassay procedure using Ag-coated beads

It was found that the PAb concentration needed to be increased (from 100 ng/ml) in the initial PAb incubation with sample (with or without MTBE) in order to achieve a desired final assay signal (≥ 1.0). A 300 ng/ml PAb concentration was selected as any further increase had little or no effect (Fig. 6.70). As found with the MTBE ELISA, optimal PAb binding occurs within 30 min.. Increased PAb incubation times produced some increase in signal, but an overlong assay. PAb-binding in a greater assay solution volume (1 ml) should be enhanced due to the constant motion of the MBs and other reagents in solution, increasing the likelihood of reagent:bead interaction. Indeed, this suggestion is supported by the results obtained, which clearly show that increased binding (and corresponding assay signals) were obtained when all assay steps were conducted on the rotator, rather than passively standing in the rack.

MTBE MB assay sensitivity and repeatability

Sensitivity of the MB assay was comparable to that of the MTBE ELISA, with an IC₅₀ (mid-point of the linear part of the sigmoidal curve) of ~5 ppm (Fig. 6.76). Sensitivity to MODMO (Fig. 6.76) with the MB assay also corresponded to that found with the ELISA, with an IC₅₀ of ~50 ppb. When a range of MODMO solutions were tested by both the MTBE ELISA and the MB assay and the results for each concentration compared (Fig. 6.77) and then combined (Fig.

6.78, the CVs for each point were <0.4%, suggesting excellent correlation between the two formats.

Summary

Overall, the transposing of the MTBE ELISA to the MB format was successful, with an IC₅₀ of 5 ppm MTBE and 50 ppb for MODMO. However, there are some factors to be considered with regard to cost: benefits for transposing the ELISA into a magnetic bead format, as follows:

- There was no significant reduction in the amounts of reagents used. In fact, using MBs increased overall assay costs and the preparation and conjugation steps (to Ag) also involved additional reagents and increased preparation time.
- No significant reductions in assay times. In fact, the length of time necessary for Ag-bead coating (36 hr) is much greater and much more complex than Ag-coating of 96-well ELISA plates (12 hr). However, should long-term stability of stored Ag-coated beads be acceptable, then greater quantities can be prepared in advance. Indeed, there are commercial pre-coated beads on the market (Dynabeads).
- Each Dynal magnetic rack is limited to 6 sample tubes. Therefore, if only 2 replicates are acceptable per sample, this still only allows for only 2 samples to be tested (plus a blank control). Similarly, no more than 2 racks can be practically used at a time, due to the 12-tube limitation of the Dynal rotator. However, it is possible to upgrade this procedure to use larger capacity magnetic racks.
- The MB immunoassays are technically more difficult to perform, each requires accurate, sequential, manual pipetting applications of reagent/WB individually, tube by tube, through all stages of the assay. Whilst this can also be true for ELISA plates, a number of automated devices are available for rapid, automated ELISAs (such as automated plate washers).

Therefore, it has been proven as a proof of principle that this MB MTBE assay works and still remains an option, but as pointed out above, a better assay design from the side of things could be improved. Similarly, the production process for coated beads could be improved. However, once adapted, the MB assay could clearly accommodate more samples to be simultaneously assayed, which is extremely advantageous for field screening. This concludes the immunoassay development work in this thesis. Chapter 7 summarises the conclusions gained from this work and suggestions for future developments are also included.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 Conclusions

The work in this thesis has concerned the development of an integrated early-warning system for the detection of fuel, which has leaked into groundwater and soil. The detection process is intended to occur 'in the field', close to the source of the leak, rather than by way of sending environmental samples to a centralised laboratory for analysis – a process which often incurs the following disadvantages (1.3.4.5):

- (1) Excessive sample turnaround time
- (2) Excessive sample size.
- (3) Sample instability.

To this end, the work has focussed on an integrated detection method based on a two-step technological approach:

- (1) An array of novel sub-surface reflectometric sensors buried beneath and around a UST, which will trigger a warning signal to a distant monitor should a fuel leak be detected.
- (2) A novel immunoassay for MTBE – an ubiquitous petrol oxygenate, which is highly water-soluble and is consequently a very mobile environmental contaminant, which can be used as a marker of the outer limit of a fuel contamination incident. MTBE also poses a major threat to global groundwater and drinking water resources, it is highly recalcitrant and has a very low taste/odour detection threshold (15-45 ppb). Currently, there are no commercially-available comparable field tests for MTBE.

Three formats were investigated: enzyme-linked immunosorbent assay (ELISA), enzyme-linked immuno flow-through assay (ELIFA) and magnetic bead assay.

Work on the first method began with an extensive investigation and reappraisal of two existing reflectometric sensor technologies, which resulted in a redesigned composite sensor, which could be part of an integrated subsurface sensor array (Chapter 2). Further development work culminated in a critical appraisal of the efficacy of these sub-surface sensors and a re-evaluation of the way in which they can be best used to monitor for fuel leaks, particularly around

underground storage tanks (USTs). It was initially envisaged that subterranean sensors would be placed subsurface in a net or grid formation around at-risk sites, which would then monitor the immediate area for fuel leaks and act as an unattended and remote early warning system. This plan was investigated in both laboratory and lysimeter trials. The sensors, with associated data collection and transmission technologies, were deployed in a subsurface network, which was successfully used to identify fuel leaks and plume movement. This system has considerable advantages over existing methods for monitoring USTs (tank gauges and monitoring wells), which may not be sensitive to extended and slow leakage (1.4.1-1.4.2).

However, the work showed that the vagaries of fuel leaks and subsequent fuel plume formation and movement could pose problems with regard to practical deployment around a real UST. Petroleum plumes form and flow according to very site-specific conditions concerning the soil characteristics, soil water content and groundwater flow. Therefore, any formalised sensor placements around existing leaking USTs, or new USTs, are likely to be subject to regional and site-specific variations, which may be difficult to predict, and which may change over time. Put simply, it is relatively easy for the sensors to 'miss' a leaking fuel plume, which may be fairly close to them. However, the sensors were designed to be reasonably low-cost and so a sufficient number can be deployed at each site. Also, it may be possible to make use of known geological data and groundwater flow patterns in order to predetermine the general direction of any leaking plume and aid appropriate siting of the sensors. Placement of sensors could also beneficially influence preferential flow, thereby increasing likelihood of free-phase detection.

It is consequently suggested that the mere scattering of sub-surface sensors around a UST is not sufficient for a reliable early warning system for fuel leaks. What is essentially needed is a collective sump (or bund), placed beneath the UST, which would guide any leakage to a critically placed sensor. Therefore, it is suggested that the sensors could be incorporated into future designs of secondary containment tanks/skins or bunds, as suggested in Chapter 4. Clearly this would require collaboration with UST manufacturers and a subsequent UST/sensor development project. However, sensors could also be deployed underneath the bunds. Additionally, they can be used for monitoring above-ground USTs and pipework.

The competitive MTBE immunoassay was successful in both ELISA and magnetic bead format, with an IC₅₀ of 5 ppm for both, although the latter involved a greater degree of handling expertise for the end-user. Therefore, the MTBE assay developed in this work can be used as an field-based screening tool, which can allow an operator to identify and track the extent of a fuel contamination incident (by testing water samples) and enable real-time management of the

polluted area. By tracking the MTBE plume, this could potentially provide the site operator with an early indication of the bulk plume direction.

With regard to the MTBE immunoassay, there are two main choices:

- (1) An IC₅₀ of 5 ppm is acceptable for field-based MTBE screening and is comparable to a commercial immunoassay for benzene (SDI). The assay could now be developed into a commercial product. Either the ELISA or the magnetic bead format could be developed into an easy-to-use kit format.
- (2) Additionally, it may be possible to raise a better, more sensitive PAb against MODMO, which would require further screening and repeat development work. However, it should be noted that over 14,000 hybridomas were screened in order to obtain the one effective PAb used in this work (Diacclone SAS, *pers.comm*). Also, the degree of sensitivity may be inherent in the low molecular weight and chemical structure of MTBE itself.

7.2 Recommendations for future work

- Full-scale monitoring tests with the fuel leak sensors sited around a leaking UST and subsequent plume movement tracking using the MTBE immunoassay.
- Investigation and design of sensor/bund incorporation and manufacture followed by sensitivity and reliability testing of these conglomerations. A single sensor could be placed centrally in a recessed bund. Alternatively, a single sensor could be incorporated into the base of a secondary UST skin (4.4).
- Investigate alternative use of sensors as an overfill detection system in both fuel tanks and fuel transport tankers. In this case, the sensors would have to be placed at the maximum fuel volume levels and would trigger when the desired volume is reached.
- The frequency of false positives/negatives of the MTBE ELISA needs to be ascertained to ensure reliability of the proposed test kit, as failure to detect MTBE in real water samples at the claimed IC₅₀ (5 ppm) could have serious implications.
- Should the MTBE ELISA be acceptable as a field screening tool, then this will need developing into a commercial product, which would then require further Quality &

Assurance testing (including a wider range of real samples) and independent validation by a reputable body. The ultimate accolade would be to gain official acceptance by the EA/US EPA.

- The magnetic bead MTBE assay format was successful in the fact that results matched those obtained by the ELISA (IC₅₀ of 5 ppm). However, more work needs to be done in order to refine and simplify the assay procedure for future use as a field-test kit.
- As both TMB & ABTS colour-producing substrates are electrochemically active, an electrochemical test featuring a disposable electrode can be envisaged. Combined with the principles of capillary flow, it may be possible to deposit reagents, such as Abs/Ag-conjugate (one of which would be attached to magnetic beads) into a chamber, which is contained within the body of the disposable plastic electrode structure, but at some distance from the electrodes themselves. The sample is introduced into this chamber, where binding occurs. By then using a movable magnet, the bound beads could be drawn through a capillary channel (containing colour-producing substrate) to where the electrodes are situated. This would also effectively act as a washing step. The electrochemical activity of the HRP enzyme could then be measured.
- The long-term stability of coated magnetic beads (whether primary antibody or antigen) and other reagents will need to be determined.
- A simple handheld lateral flow-through device can also be envisaged for a one-shot, disposable test for MTBE contamination, based on the ELISA/ELIFA work, but much more research is required to find a suitable membrane support material and to overcome the problems of NSB. An ever-increasing number of membranes are being developed, which may prove more successful in the future.

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APPENDICES

Appendix 1. Technical specifications of the Medsci Data Logging Unit (Medsci Ltd., Harpenden, UK)

Data acquisition	• individual sensor input conditioning • mixed sensor type capability • multi-channel multiplexed input (expandable up to 8 channels) • 0-bit A/D conversion
Data transfer	• embedded Internet connectivity • standard PPP, TCP/IP Internet protocols • SMTP/MIME send e-mail capability • POP3 receive e-mail feature • multiple recipient e-mail address support
Telecommunications	• dual-band 900/1800 MHz GSM data compatible • SMS messaging function (alarm alerts) • integral external antenna
Control	• single 8-bit CMOS micro-controller • reprogrammable 'flash' memory • software controlled real-time clock • internal serial communications programming interface (RS232 compatible)
Power	• rechargeable battery (8 V, 4.5 Ah) • dc regulated system supplies (+5 V) • switchable sensor supply (+5 V)
Enclosure	• external dimensions 170x140x100 mm • weatherproof • impact resistant • contains; single printed circuit board, battery • external power/charger socket • external multi-way cable to sensor junction box

Appendix 2. Membrane specifications (evaluated for the sub-surface sensor)

Biodyne Nylon 6,6	Solid phase membrane assays. Solvent compatible. Intrinsic hydrophilicity. A – amino & carboxyl groups B – ammonium groups. Positively charged for ionic binding
Emflon PTFE membrane/tape	Poly tetrafluoro ethylene polymer. Repels low-surface tension liquids, wide chemical compatibility
Fluorotrans PVDF	Hydrophobic polyvinylidene difluoride (CH ₂ CF ₂), solid phase support binds proteins via hydrophobic interactions. Enables detection with common staining agents, high tensile strength & chemical resistance
GHP450 (GH polypro)	Universal treated polypropylene membrane, fluorescent detection of analytes possible
Mycelx	MYCELX is a patented polymeric surfactant technology. Bonds to hydrocarbons etc. to make them hydrophobic and viscoelastic thus removing them permanently from the water stream. www.mycelx.com
Presense	Hydrophilic polyethersulfone, high tensile strength, enhanced reflectance
Versapor R membrane	Treated acrylic co-polymer nylon support membrane, 'Fluororepel' treated for superior hydrophobicity, wide chemical compatibility

- All membranes (except Mycelx) were obtained from Pal Europe Ltd. (Portsmouth, UK). www.pal.com

Appendix 3. Salzburg groundwater analysis

WASSERANALYSE für			Nr.:	1646/03	1706/03
				Quelle Fürstenbrunn	Moorwasser
					dem Leopoldskroner Moor
Probenahme:					
Datum der Probenahme				13/08/2003	20/08/2003
Wetter				heiter	heiter
Lufttemperatur			°C	31.0	24.0
Datum der Untersuchung				13/08/2003	20/08/2003
Farbe	DIN EN ISO 7887	Hazen		gelblich	gelb
Geruch	sensorisch			modrig	modrig
Geschmack	sensorisch				
Bodensatz	sensorisch			keiner	braune Flocken
Wassertemperatur	DIN 38404-3	°C		6.0	
Desinfektionsmittel		mg/l			
Leitfähigkeit bei 20°C	DIN EN 27 888	µS/cm		146	522
SAK 436 nm	DIN EN ISO 7887	1/m		1.587	2.734
SAK 254 nm	DIN 38 404-3	1/m		13.073	46.021
UV - Durchlässigkeit (10 cm	berechnet	%		4.93	0.00
Trübung	DIN EN ISO 7027	FTU		1.18	8.55
pH - Wert	DIN 38 404-5			7.77	6.97
Säurekapazität bis pH 8,2	DIN 38 409-7	mmol/			
Säurekapazität bis pH 4,3	DIN 38 409-7	mmol/		1.65	5.91
Gesamthärte	DIN 38 409-6	°dH		4.77	17.22
Karbonathärte	berechnet	°dH		4.63	16.58
Calzium	DIN EN ISO 14 911	mg/l		31.73	103.75
Magnesium	DIN EN ISO 14 911	mg/l		1.62	11.70
Eisen	DIN 38 406-1	mg/l		0.01	1.20
Mangan	DIN 38 406-2	mg/l			
Ammonium	DIN 38 406-5	mg/l		0.00	1.19
Natrium	DIN EN ISO 14911	mg/l		0.36	3.91
Kalium	DIN EN ISO 14911	mg/l		0.11	4.81
Summe Kationen		mval/l		1.72	6.55
Chlorid	DIN EN ISO 10 304-1	mg/l		0.51	4.67
Sulfat	DIN EN ISO 10 304-1	mg/l		1.12	11.97
Nitrat	DIN EN ISO 10 304-1	mg/l		3.21	1.81
Nitrit	DIN EN 26 777	mg/l		0.000	0.040
Fluorid	DIN EN ISO 10 304-1	mg/l		0.01	0.12
Kieselsäure	DIN 38 405-21	mg/l		0.59	17.47
Orthophosphat	DIN EN 1189	mg/l		0.00	0.24
Summe Anionen		mval/l		1.74	6.33
KMnO4 - Verbrauch	EN ISO 8467	mg/l		14.38	39.03
TOC	DIN EN 1484	mg/l		2.87	10.68
Sauerstoffgehalt	DIN EN 25814	mg/l		11.37	
Sauerstoffsättigung	berechnet	%			
Aliphat.Kohlenw.	DIN 38 409-18	mg/l			
Halogenkohlenw.	DIN EN ISO 10301	mg/l			
Koloniezahl in 1 ml bei 22 °C	Membranfiltration				
Koloniezahl in 1 ml bei 37 °C	Membranfiltration				
Coliforme Keime in 100 ml	Membranfiltration				
Escherichia coli in 100 ml	Membranfiltration				
Enterokokken in 100 ml	Membranfiltration				

Ortsbefund:
 Beurteilung:

Appendix 4. List of patents, proceedings and publications made during this thesis.

PATENTS

Huntley, S., Setford, S., Saini, S., Vermot-Desroches, C. & Wijdenes, J. (2004) *Immunochemical methods and reagents for measurement of fuel oxygenates*. Patent No: 0325114.7

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