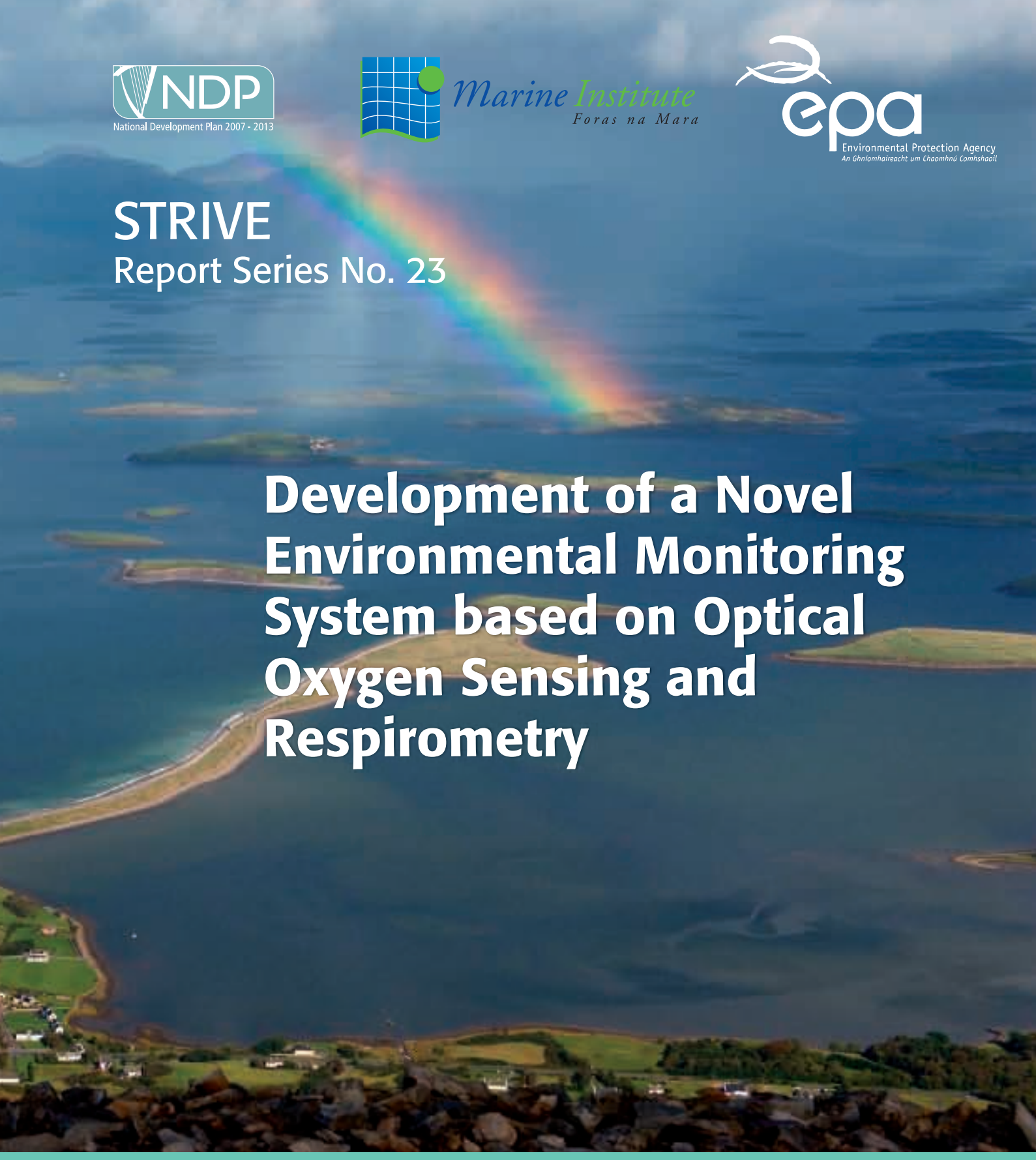

National Development Plan 2007 - 2013

Marine Institute
Foras na Mara

Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

STRIVE
Report Series No. 23

Development of a Novel Environmental Monitoring System based on Optical Oxygen Sensing and Respirometry

SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Oidhreacht agus Rialtas Áitiúil
Environment, Heritage and Local Government

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The Environmental Protection Agency (EPA) is a statutory body responsible for protecting the environment in Ireland. We regulate and police activities that might otherwise cause pollution. We ensure there is solid information on environmental trends so that necessary actions are taken. Our priorities are protecting the Irish environment and ensuring that development is sustainable.

The EPA is an independent public body established in July 1993 under the Environmental Protection Agency Act, 1992. Its sponsor in Government is the Department of the Environment, Heritage and Local Government.

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Marine Institute / Environmental Protection Agency Partnership:

Advanced Technologies for Monitoring Water Quality

STRIVE Programme 2007–2013

Development of a Novel Environmental Monitoring System based on Optical Oxygen Sensing and Respirometry

(AT-04-01-01)

Final Report

Prepared for the Marine Institute and the Environmental Protection Agency

by

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DISCLAIMER

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FOREWORD

The Environmental Protection Agency (EPA) and the Marine Institute entered a strategic partnership agreement in July 2005 in the broad areas of Environmental Technologies and Water-Quality Monitoring. The aim was to catalyse an innovative programme of environmental technology research to underpin the development of the Smart Green Economy.

The specific aims of the partnership were to:

- Build national research and innovation capacity in the area of water-quality monitoring, particularly in respect to the implementation of the Water Framework Directive.
- Provide technological support for the sustainable development of aquatic/marine resources.
- Support the creation of new industrial capabilities in these areas.

An initial core suite of three-year research projects was funded with the objective of forming a consortium of national capabilities to address market opportunities associated with marine and environmental technology development. A review of the projects and the overall programmatic approach indicates that the performance and achievement of strategic objectives are broadly in line with those established at the outset.

In addition, as the projects evolved, the ability to test and demonstrate prototype and pre-operational environmental sensors and communications technology in the field became apparent. The SmartBay pilot project emerged as a response to this and was developed jointly by the Marine Institute and the EPA under the initial collaborative agreement. The objective is to develop SmartBay (in Galway Bay) as a strategically positioned and uniquely located marine research, test and demonstration platform, with a reputation for leading-edge technologies for global markets and for the development of innovative solutions to important environmental questions. The SmartBay project is advancing with input from a wide range of agencies, researchers, industry and end users.

The EPA and Marine Institute have agreed a further collaborative research programme for the period up to 2011. Its main focus will be to support the implementation of a number of European Union (EU) Directives (Water Framework, Strategic Environmental Assessment, Marine Framework and Bathing Water) as well as national efforts in response to the EU Environmental Technologies Action Plan.

In this research report we publish the findings of one of the projects on water monitoring systems. The report presents some exciting results in terms of the quality of the research, and the expertise and capability developed from the agencies' shared investment.

In the current economic climate, cooperation between research funders is more important than ever to maximise the impact and benefits from investments in research. The partnership approach adopted by the EPA and the Marine Institute in relation to the research presented in this report is an excellent example of such cooperation and is a vital support in the development of Ireland's smart green economy. This cooperation has led not only to the development of critical national research capacities and capabilities, but will also help position Ireland as a leader in developing innovative technological solutions for the environmental and marine areas and to take advantage of one of the fastest growing markets in Europe.

Dr Mary Kelly
Director General
Environmental Protection Agency

Peter Heffernan
Chief Executive
Marine Institute

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Executive Summary

In this project the team – comprising academic and industrial partners – has developed a new system for toxicological monitoring of environmental samples. The new system, which can be used particularly for wastewater, contaminated freshwater and seawater samples, is based on a number of alternative biological models for toxicity testing, employs optical oxygen respirometry as a method of detection. Compared with existing tests and systems for toxicological assessment, this approach provides high sensitivity and specificity because of its measurement of sub-lethal changes in the metabolism of test organisms, along with the method's high sample throughput, miniaturisation, affordable cost and the general convenience provided by the optical respirometry. The system operates with dispensible O_2-sensing probes, simple and robust assay procedures, standard microtitter plate assay platforms and widely available measurement instrumentation (fluorescent plate readers).

A significant development of this technology, its application to various biological models, chemical and environmental samples and extensive validation of the panel of new toxicity assays were undertaken under this project. This system for toxicological assessment has been developed using a panel of convenient and ethical biological models – ranging from common prokaryotic and eukaryotic cell lines and small invertebrate and vertebrate aquatic organisms – which can be used individually or bundled together. Animal models tested include *Daphnia magna* which is regarded as one of the golden standards in traditional toxicity testing of chemical and water samples, and other organisms, many of which are actively used for the analysis of gene and protein function, various disease states and drug development. Currently, rather simple but time-consuming and not very objective mortality/immobilisation based assays are used with *Daphnia* and also the other animals, which are prone to false-positive results and have limited scalability whereas the new respirometric assays have provided automation, increased sample throughput and general convenience. The new toxicity assays were established, optimised and evaluated with toxicants and chemicals of different type, including heavy metal ions, pesticides, polyaromatic hydrocarbons (PAHs), organic solvents, marine toxins, pharmaceuticals, drugs, and also mixtures of chemicals. The assays were subsequently validated with real environmental samples (including contaminated water, wastewater, water discharged from wastewater treatment plants [WWTP]), and benchmarked against the standard toxicity tests currently used by environmental laboratories. In many cases, they provided higher sensitivity to the toxicants, and allowed the detection of sublethal toxic effects which cannot be picked by conventional tests. Therefore, such assays are of particular relevance to biochemical toxicology and environmental monitoring. Several pilot trials were conducted in collaboration with several environmental and toxicological monitoring laboratories in Ireland and Europe.

The project work has generated a large amount of new experimental and toxicological data and these results have provided the basis for a significant number of scientific publications in leading international environmental and bioanalytical journals, and for one patent application. All project objectives and milestones have been met. Towards the end of the project, the team has engaged in discussions with the Marine Institute and the Environmental Protection Agency (EPA) and our industrial partner Luxcel Biosciences in relation to the larger-scale trials with potential users of this monitoring and screening system; its wider deployment in relevant laboratories and organisations in Ireland and abroad; and other opportunities for dissemination of the new sensor technology.

1 Introduction

Globally, approximately one-third of available freshwater is currently used for agricultural, industrial or domestic purposes. This results in a contamination of the water with a wide range of pollutants originating from ~300 million tonnes of compounds used in industrial and consumer products, ~140 million tonnes of fertilisers, several million tonnes of pesticides and 0.4 million tonnes from oil and gasoline spillages (FAO, 2006). To tackle the emerging threat of contamination and depletion of freshwater stocks, large initiatives such as the Clean Water Act (CWA) in the United States (Congress, USA, 1977) and the European Union Water Framework Directive (WFD) (OJL, 2000) have been established. The CWA aims at 'restoring and maintaining the chemical, physical, and biological integrity of the Nation's waters', whereas the WFD is concerned with the 'scope of water protection to include all waters, to set clear objectives in order that a "good status" be achieved'. The successful realisation of such projects, and of the other environmental monitoring tasks, is linked to the availability of techniques for detailed toxicological assessment, screening and monitoring of a large number of chemical and environmental samples, plus the validation and wide deployment of such techniques.

Water-quality monitoring programmes exist in many of the Member States throughout the European Union (EU). With the implementation of the Water Framework Directive (WFD, Council Directive 2000/60/EC) (OJL, 2000) all Member States must harmonise their national monitoring methods for each common metric (parameter indicative of a biological water quality element) used to determine the state of the aquatic environment to ensure consistent and comparable classification results for all biological community quality elements used (WFD Annex V, 1.4.1).

The recent Environmental Protection Agency (EPA) water quality assessment (EPA, 2007) for Irish rivers and streams shows that 69% of river/stream length is categorised as 'unpolluted', 18% of streams as 'slightly polluted' and 12% of river channel as 'moderately polluted', with a further 0.6% categorised as 'seriously polluted'. These water-quality problems extend to Irish lakes, approximately 18% of which were classified as 'eutrophic' or 'hypertrophic' and exhibited varying signs of pollution together with the potential impairment of their beneficial uses in the 2001–2003 period.

Environmental monitoring, including monitoring of wastewater and contaminated fresh and seawater samples, is therefore one of the priority areas in national and EU research programmes. Monitoring encompasses a wide range of technologies, including a variety of toxicity testing/risk-assessment methodologies, some of which involve the use of higher animals such as mice. Testing for individual pollutants is also common, yet in many cases real-world toxicology demands detection of multiple contaminants, or registration of a problem stemming from unknown sources.

The new approach to toxicity screening proposed in this project addresses ethical and economic issues of current environmental monitoring techniques. It provides a new highly efficient and innovative methodology and corresponding bioanalytical and sensing platforms and solutions, which will complement or even replace established monitoring techniques, many of which are tedious, inefficient, inadequate and/or expensive.

The main requirements for the new monitoring system developed under the project were formulated as:

- The use of simple, inexpensive and ethical biological models – small aquatic animals such as *Daphnia magna*, brine shrimp *Artemia*, zebrafish (*Danio rerio*) embryos/juveniles, soil worms (*Caenorhabditis elegans)*, common prokaryotic and eukaryotic cell lines (mammalian and bacterial cells) and enzymatic systems.

- The use of biological oxygen consumption as a generic biomarker of toxicity and as a parameter used for the quantification of toxicological impact or hazard of environmental samples.
- The use of new detection methodology – optical oxygen respirometry recently developed at University College Cork (UCC), which provides the capability of parallel toxicological assessment of large numbers of environmental and water samples, along with high sensitivity and convenience.
- The use of simple, robust, widely available instrumentation, analytical tools and integrated solutions, which would allow miniaturisation, automation and cost saving, which do not require specially trained staff and sophisticated facilities and can operate both in centralised laboratories and in field conditions.
- The measurement of different read-out parameters to allow a detailed assessment of toxicity of environmental samples, mechanistic studies and to facilitate the analysis of mixtures, combinatorial effects, and identification of toxicants in unknown samples.

This project also aimed at addressing the increasing requirement for the setting-up of centralised and small laboratories, screening groups and mobile services. These will use the new techniques and analytical and toxicological platforms developed in the project, which can complement the existing (but often sophisticated and overstretched) centralised facilities, to conduct environmental monitoring, in order to perform screening and monitoring of water samples at various sites including remote locations, both within Ireland and abroad.

Once developed and established in the research laboratories of project participants, the new toxicological screening system(s) will need to be validated extensively with different model organisms and a significant number of reference toxicants of different type, their mixtures, and finally with real environmental samples, including wastewater, river and seawater and industrial effluents.

Extensive in-house and external testing and validation of the new environmental monitoring systems developed in the project will enable their broader use in different locations and for various toxicological tasks, thus allowing rapid dissemination and commercialisation of project results. It was anticipated that the project's commercial partner – the Irish biotech company Luxcel Biosciences, which was actively involved in project work –– would be the main driver of the independent validation, dissemination and commercialisation of the results. It was also expected that validation and dissemination would be augmented by the extensive scientific, technical and industrial partnerships of the academic researchers and organisations involved in this project.

Altogether, the project aimed at generating a large amount of new knowledge in the new areas of environmental monitoring and sensor research; the creation of advanced screening platforms employing the optical oxygen sensing principles, and their implementation as working prototypes; and the development of integrated systems and solutions for toxicological monitoring of marine and environmental samples (mainly contaminated water and toxicants present in the aqueous environment).

The original concept of this project and working flow chart are shown in Figure 1.

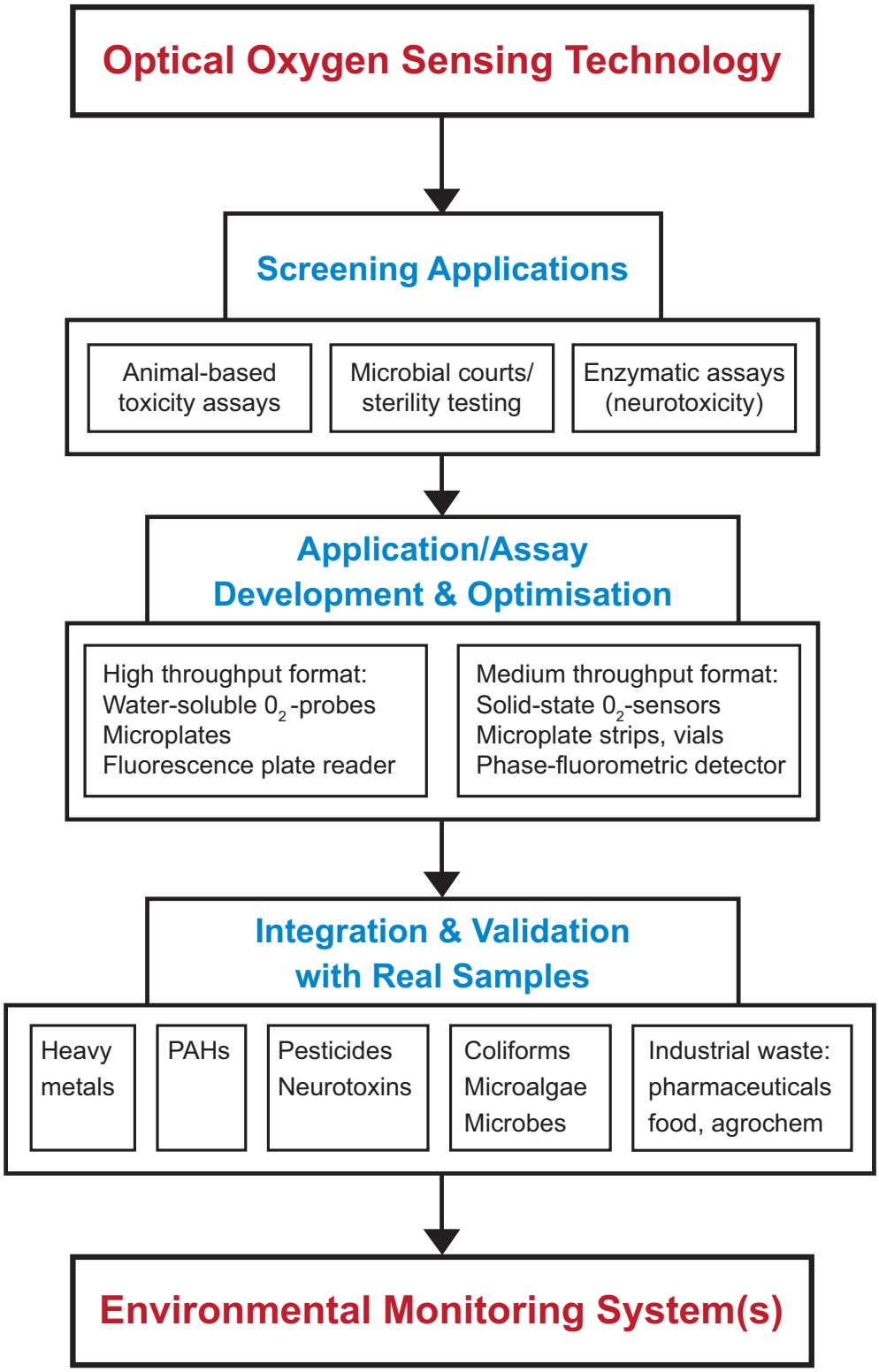


Figure 1: Overall concept of the Oxygen Sensing Project and proposed work flow chart. (PAHs = polyaromatic hydrocarbons)

2 Results

2.1 Optical Oxygen Respirometry – A New Method of Toxicological Assessment and Screening

Molecular oxygen (O_2) and O_2 consumption rates are universal and sensitive biomarkers of the general viability of aerobic cells and organisms and their metabolic responses to various stimuli. A new methodology for the monitoring of physiological/metabolic responses of small organisms, cells and enzymes via changes in their oxygen respiration was developed by the project team (Papkovsky et al., 2006; O'Mahoney et al., 2005). This approach employs quenched-phosphorescence oxygen sensing using water-soluble phosphorescent O_2 probes added to the sample and detection on standard instrumentation – fluorescence readers and spectrometers. These probes possess longwave, long-decay emission and can be detected with high sensitivity and selectivity on standard and time-resolved fluorescence spectrometers and plate readers (Fig. 2 and Table 1). Their phosphorescence (both intensity and lifetime) is dependent on O_2 concentrations, thus allowing O_2 quantification and kinetic monitoring.

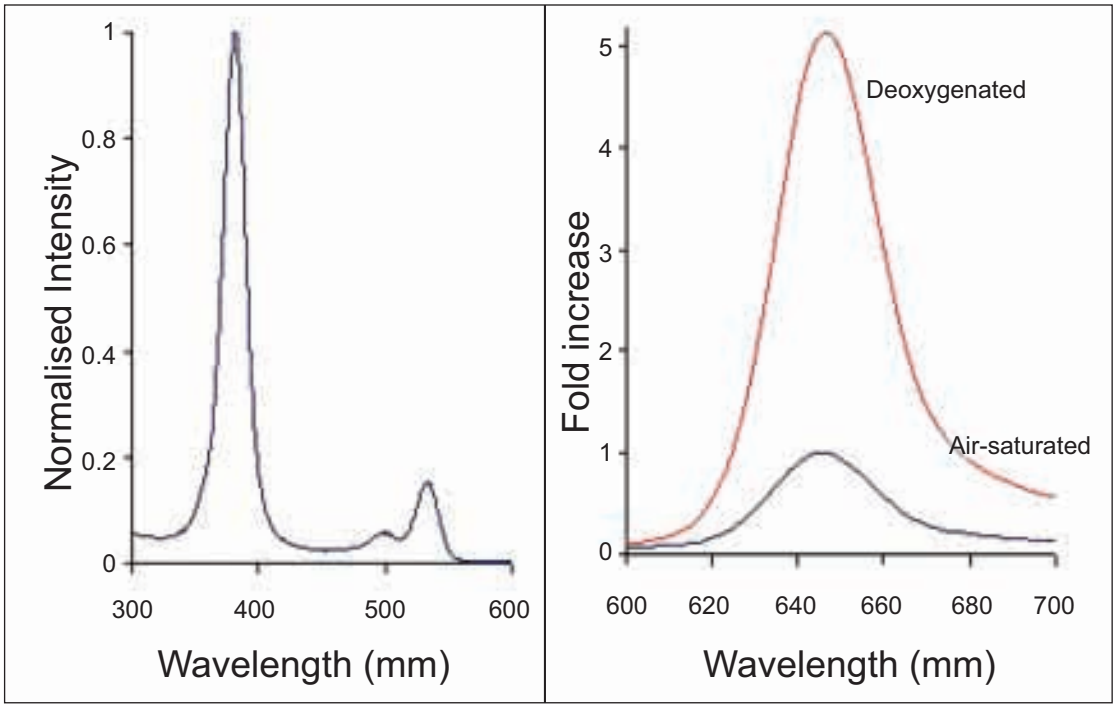
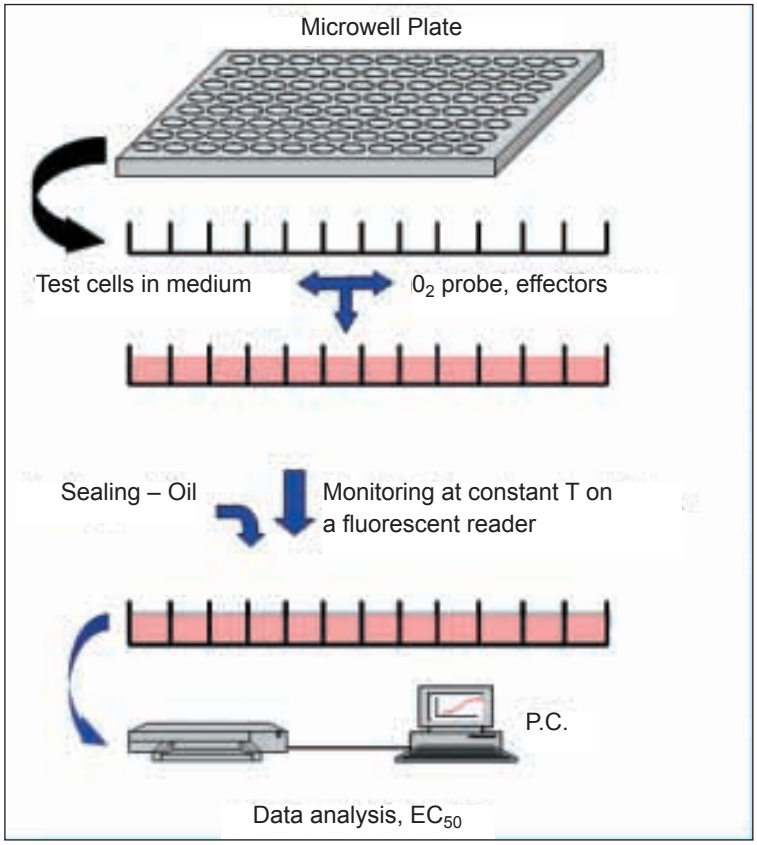


Figure 2: Spectral characteristics of the phosphorescent O_2-sensing probe used in this project.

Table 1: Signals measured using different instruments or detection modes.

Detection mode	Blank	Normoxia (21% 0_2)	Anoxia (0% 0_2)	Signal/Blank
Steady-state fluorescence	>50 IU	~ 200	~ 600	2-6
Time-resolved fluorescence	<500 cps	> 100,000	~ 1,000,000	Hundreds
Lifetime measurements	n.a.	~ 25ms	~ 90 ms	n.a.

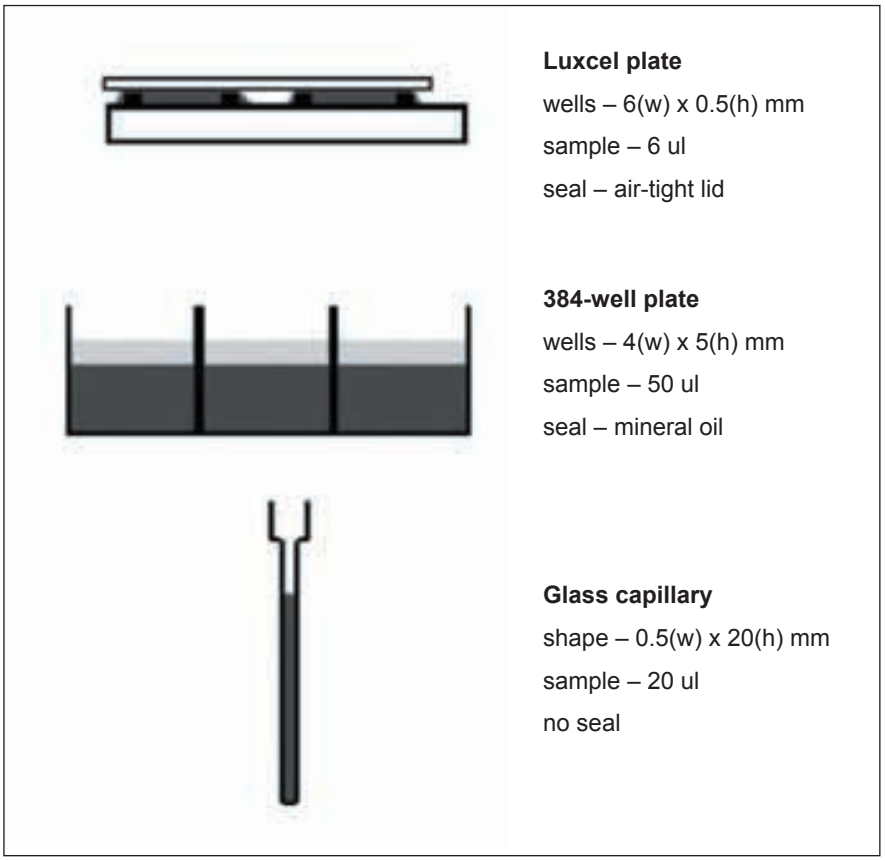
The team developed several different measurement formats, including: (i) standard 96/384-well plate with oil seal, (ii) the low-volume sealable 96-well plate developed by Luxcel Biosciences and (iii) the capillary cuvette, which gives researchers flexibility and versatility in conducting different assays and measurement tasks, particularly toxicological assessment with different biological models. These assays provide simple, non-invasive, monitoring of large numbers of biological samples and rapid, sensitive assessment of alterations in their oxygen consumption and metabolism. They are well suited to screening for acute toxicity of compound libraries and environmental samples, and the study of animal physiology and metabolism, allowing simple, high throughput assessment and determination of sub-lethal effects, EC_{50} values, modes of toxicity and biological hazard. The general procedure of such screening assays is shown in Figure 3, and the schematic representation of the three main measurement formats and standard experimental set-up are shown in Figure 4.



- Prepare test organisms.
- Expose to samples.
- Add 0_2 probe.
- Seal (e.g apply oil).
- Monitor fluorescence at T= Const.
- Analyse signal profiles.

Figure 3: General scheme of the assessment of toxicants using model organisms and optical oxygen respirometry.

(a)



(b)

Figure 4: The three main measurement formats developed in the project (a) and a typical experimental set-up (b) for the measurements on a standard fluorescent reader using phosphorescent oxygen probes, model organisms and optical oxygen respirometry.

In the project, this optical oxygen respirometry technique and measurement formats were applied to develop a panel of new screening assays and alternative biological toxicity testing systems. In particular, the following animal models and oxygen-dependent biological systems were investigated:

- **Invertebrate organisms:**
  - *Daphnia magna* – widely used in traditional aquatic toxicity testing.
  - *Artemia salina* – brine shrimp, potential for toxicological.
  - *Caenorhabditis elegans* – soil worms, classical model for genetic studies.
- **Vertebrates:**
  - *Danio rerio* (zebrafish) embryos/juveniles.
- **Prokaryotes/microorganisms:**
  - *Escherichia coli.*
  - *Vibrio fisheri.*
  - *Pseudomonas.*
- **Eukaryotic cells:**
  - Jurkat T cells – human T cell lymphoma.
  - PC12 – neuroseretory cells.
  - HepG2 – human liver cells (hepatoma).
  - HCT116 – human breast cancer cell line.
  - Hepatocytes from rat liver.
- **Oxygen-dependent enzymes:**
  - Glucose oxidase – as a simple model for method development and optimisation.
  - Coupled cholineesterase/choline oxidase system inhibited by neurotoxins and pesticides.

These bioanalytical systems, individually or in combination, were applied to the following groups of toxicants, measurement and toxicological tasks:

- **Cell and animal-based toxicity assays:**
  - Heavy metal ions: Cd^{2+}, Zn^{2+}, Co^{2+}.
  - Organic solvents (volatile) – Benzene, Toluene, Styrene. CS_2, Chloroform.
  - PAHs – naphthalene, pyrene.
  - Pesticides – aroclor, parathione, paraoxon, carbofurane.
  - Marine toxins – microcystin-LR (MCLR).
  - Food toxins – aflatoxin.
  - Classical mitochondrial inhibitors and uncouplers – rotenone, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), antimycin A, oligomycin, azide.
  - Mixtures of toxicants – PAHs and pesticides.
- **Analysis of environmental samples:**
  - Industrial effluents and wastewater from WWTP.
  - Marine samples and algal blooms.
- **Analysis of microbial contamination:**
  - Testing of sterility and microbial load in water, broths, media, industrial waste.
  - Total aerobic viable counts in raw meat – food-safety assessment, process control.
- **Mechanistic studies of toxicity:**
  - Microcystins.
- **High-sensitivity test for microcystins using mammalian cell lines (HepG2, Jurkats)**
- **Highly sensitive enzyme-based neurotoxicity screen:**
  - Paraoxone.
  - Carbofurane.

In addition, a new technique to enable the real-time monitoring of metabolic responses of mammalian cells was developed. This is based on dedicated *intracellular* O_2 probes which are loaded passively into the cells and then monitored by time-resolved fluorescence. This approach allows for the monitoring of local O_2 concentrations within the cell and changes of this parameter (linked to the oxygen-consumption rate) in response to stimulation

or toxicological impact. This technique, which allows the monitoring of rapid, transient metabolic responses, was demonstrated successfully with a number of common cell lines, including PC12, HepG2, Jurkat, HCT116 and 3T3. Subsequently, it was applied to the studies of perturbed cell metabolism by classical mitochondrial inhibitors and uncouplers, sustained plasma membrane depolarisation (in PC12 cells), other drugs and toxins.

A summary of results of these studies and representative experimental data are presented in the sections that follow. For more detailed information and scientific references related to this project, please refer to the scientific publications emanated from this project listed in Section 4.2.

2.2 Detection of Aerobic Microorganisms by Optical Oxygen Respirometry

A simple assay has been developed for the determination of aerobic bacteria in complex samples such as broth, industrial wastewater and food homogenates (O'Mahoney and Papkovsky, 2006). This employs commercial phosphorescent oxygen-sensitive probes to monitor oxygen consumption of samples containing bacteria using standard microtitter plates and fluorescent plate readers. As bacteria grow in aqueous medium, at certain points they begin to deplete dissolved oxygen, which is seen as an increase in probe fluorescence above the baseline signal. The time required to reach the threshold signal is used to either enumerate bacteria based on a predetermined calibration or to assess the effects of various effectors on the growth of test bacteria in comparison with an untreated control. This method allows for the sensitive (down to a single cell), rapid (0.5 to 12 h) enumeration of aerobic bacteria without the need to conduct lengthy (48 to 72 h) and tedious colony counts on agar plates. It also allows for the screening a wide range of chemical and environmental samples for their toxicity. These assays have been validated with different bacteria, including *Escherichia coli*, *Micrococcus luteus*, and *Pseudomonas fluorescens*, with the enumeration of total viable counts in broth and

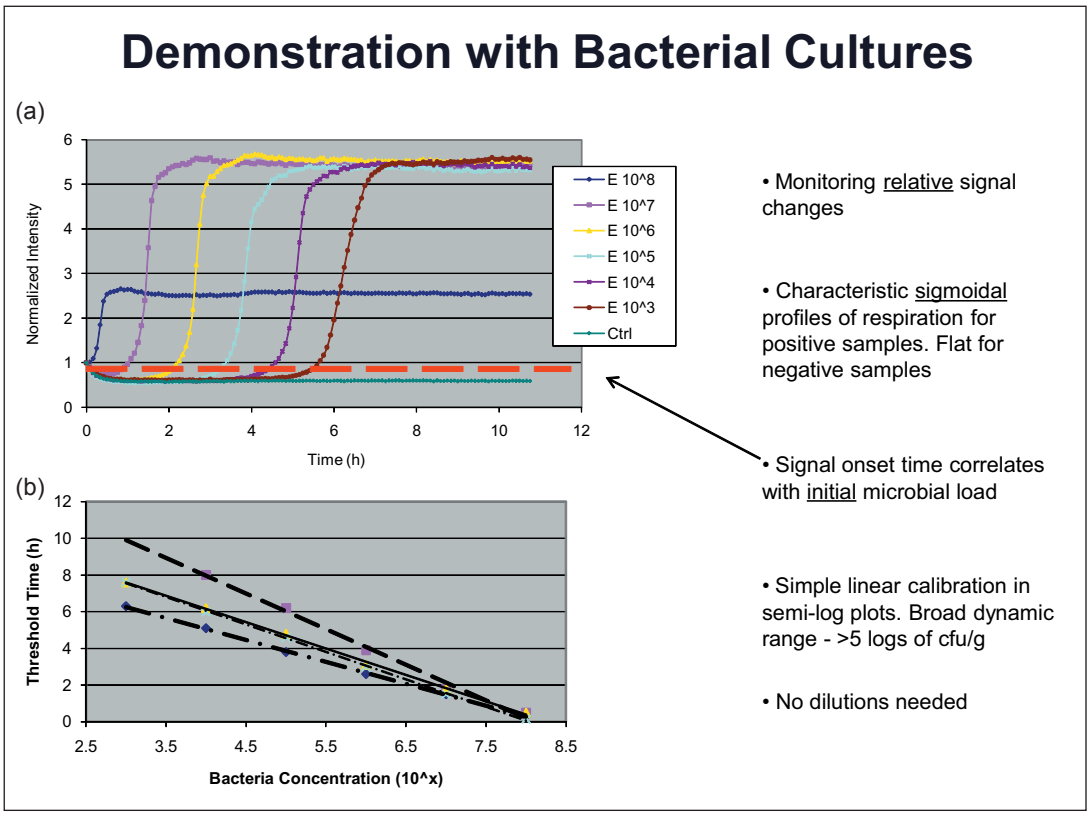


Figure 5: Typical respiration profiles for *E. coli* for different initial cell numbers (a) and calibration functions for different bacteria (b): *E. coli* (blue), *S. aureus* (pink), *K. aerogenes* (yellow) and *P. rettgery* (blue crosses).

Table 2: Calibration equations for the enumeration of *E. coli, P. fluorescens* and *M. luteus* in nutrient broth obtained in different experiments (including repeats).

Bacteria, Assay temperature	Analytical equation*	R^2
***E. coli*, 37 °C**	t_{onset} = -0.5235 Ln (Conc.) + 11.807	0.9939
***E. coli*, 30 °C**	t_{onset} = -0.6993 Ln (Conc.) + 14.403	0.9899
***M. luteus*, 30 °C**	t_{onset} = -0.7322 Ln (Conc.) + 15.36	0.9946
***P. fluorescens*, 30 °C**	t_{onset} = -1.2874 Ln (Conc.) + 24.028	0.9966

*All concentrations were measured in quadruplicates (N=4)

industrial food samples (packaged ham, chicken, and mince meat), and comparison with established agar plating and turbidimetric assays (absorbance at 600 nm) has been given. Signal onset time allows the simple determination of the initial microbial load from linear calibration (see Figure 5, Table 2).

This measurement methodology was successfully applied to the quantification of microbial contamination in industrial samples of raw meat. Figure 6 below shows good correlation with a standard total viable counts (TVC) test on agar plates.

This methodology is also applicable to the analysis of wastewater samples (total load of aerobic bacteria), sterility testing and selective assays (e.g. coliforms). Corresponding studies are under consideration by the team.

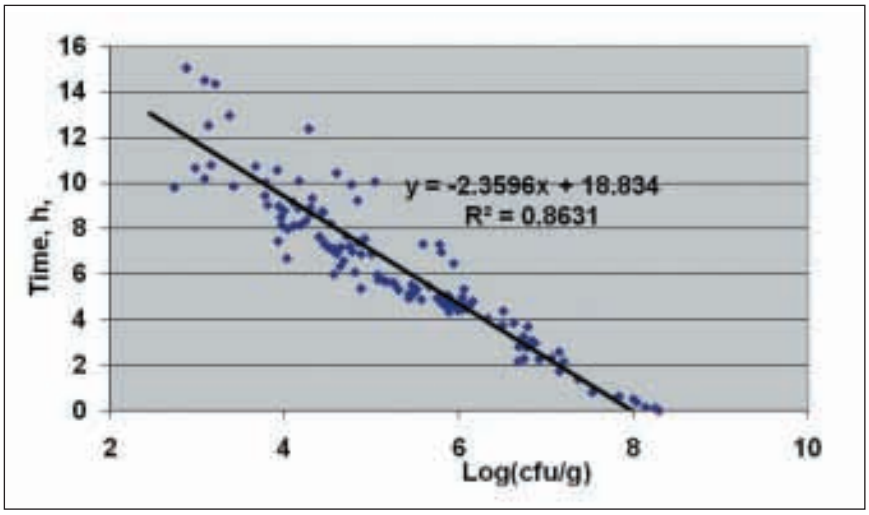


Figure 6: Calibration function and analytical equation for the determination of microbial load in samples of raw meat using respirometric assay.

2.3 Use of *Daphnia magna* as Model Organism in Respirometric Toxicity Assays

The optical oxygen sensing method was applied to monitor the respiration of individual *Daphnia magna* and to develop a simple, automated screening assay for the assessment of acute toxicity of large numbers of chemical and environmental samples (Zitova et al., 2008). Using standard microtitter plates and a fluorescent reader, *Daphnia* were exposed to the toxicants and effluent samples for 24 hours or 48 hours and then analysed for changes in respiration relative to untreated controls. Compared to the established *Daphnia* test based on mortality assessment, the new assay showed good agreement for reference toxicants including $K_2Cr_2O_7$, SLS and heavy metals, ease of generation of dose-response curves and EC_{50} values, and the ability to detect sub-lethal effects of toxicants which inhibit or activate animal respiration. In many cases, particularly with industrial effluents, the assay showed higher sensitivity and robustness. It is therefore well suited for environmental monitoring.

Representative respirometric profiles of *Daphnia magna* and toxicity data are presented in Figures 7 and 8 and in Tables 3 and 4.

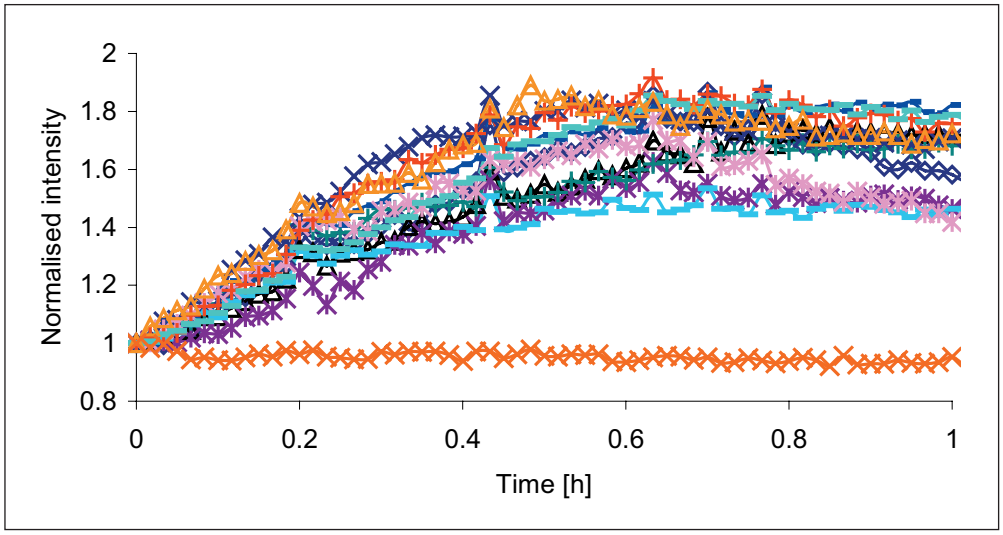


Figure 7: Respiration profiles of individual *Daphnia* measured on Luxcel plate (12 identical samples). Increase in fluorescence signal reflects gradual depletion of dissolved O_2 in test sample over time, i.e. respiration rate. Two flat lines represent negative controls without *Daphnia*.

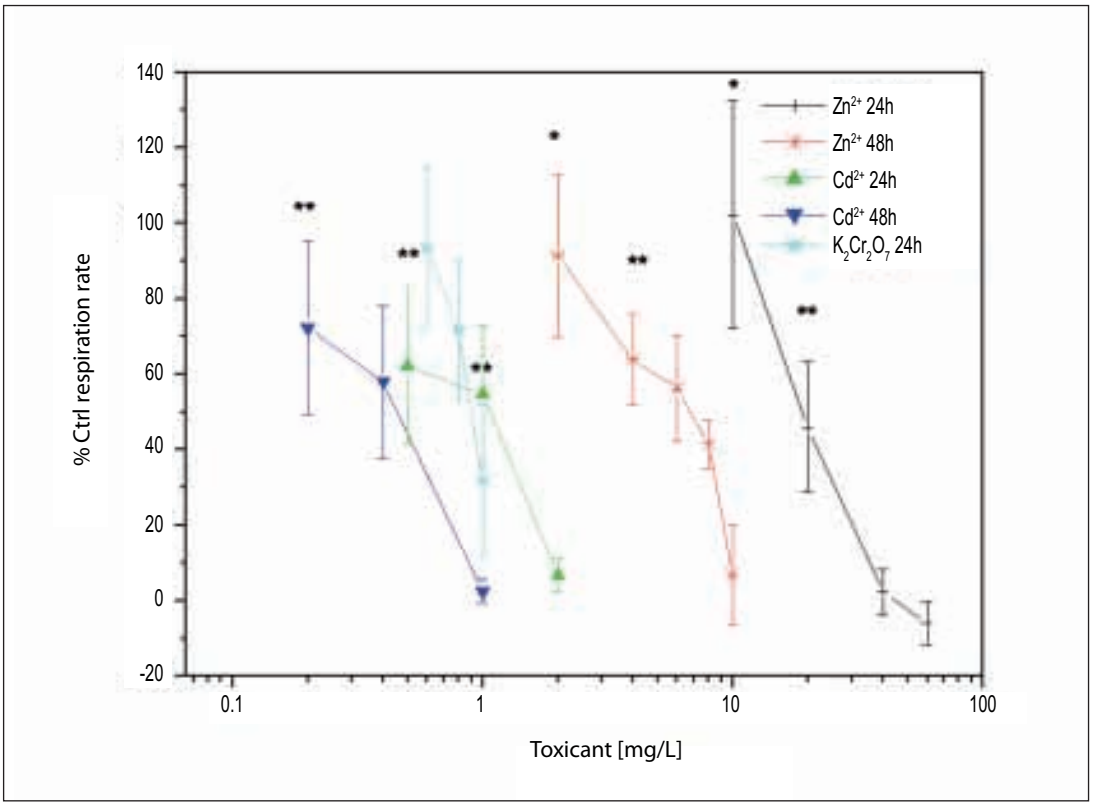


Figure 8: Dose and time dependence of toxic effects of different chemicals on *Daphnia* respiration.

Table 3: EC_{50} values for standard toxicants measured with *Daphnia* by respirometry and standard mortality test.

Toxicant	Standard assay EC_{50}-24h [mg/L]	Respirometric assay EC_{50}-24h, (c_{min}.) [mg/L]	Standard assay EC_{50}-48h [mg/L]	Respirometric assay EC_{50}-48h, (c_{min}.) [mg/L]
$K_2Cr_2O_7$	1.12 [17], 3.9 [10]	0.899±0.11, (0.8)	–	–
SLS	50 [10]	64.9±8.28, (60)	–	–
Zn^{2+}	1 [38]	4.52±0.58, (4)	0.56 [38], 1.83±0.07 [39]	1.49±0.14, (0.9)
Cd^{2+}	4.66 [38]	0.63±0.23, (0.3)	1.88 [38], (0.615±0.03) [40]	0.16±0.06, (0.08)

Table 4: Toxicity data for a panel of industrial effluents analysed.

Effluent No.	Acute toxicity test *Daphnia* (TU)	*Daphnia* EC_{50}-24h [% vol/vol] (TU)	EPA class	Activity description
1	<1	ER	5	The use of a chemical or biological process for the production of basic pharmaceutical products.
2	16.4	14.0±5.0 (7.13)	8	The manufacture of paper pulp, paper or board.
3	3.6	85.6±37.4 (1.17)	3	The production, recovery, processing or use of ferrous metals in foundries having melting installations.
4	14	19.85±3.82 (5.04)	12	The surface treatment of metals and plastic materials using an electrolytic or chemical process.
5	13.5	4.01±0.47 (24.94)	5	The manufacture by way of chemical reaction processes of organic or organo-metallic chemical products.
6	6.99	14.54±0.74 (6.88)	5	The manufacture of pesticides, pharmaceuticals or veterinary products and their intermediates.
7	2.43	14.19±6.05 (7.05)	5	The use of a chemical or biological process for the production of basic pharmaceutical products.
8	5	ER	7	Commercial brewing and distilling, and malting in installations.
9	1.3	ND	12	The manufacture or use of coating materials in processes.
10	<1	ND	5	The use of a chemical or biological process for the production of basic pharmaceutical products.

2.4 Respirometric Toxicity Assay using Zebrafish (*Danio rerio*) Embryos

Zebrafish (*Danio rerio*) has previously been established as a useful model for genetic manipulation, toxicological studies and environmental monitoring. Current analytical approaches to study toxicological effects in zebrafish employ end-point lethality assessment as well as more complex biomarkers and measurement techniques. This model animal was successfully applied to develop a sensitive, high-throughput, automated platform for toxicological assessment, (Zitova et al., 2008) by measuring respiration rates of *individual* zebrafish embryos in low-volume sealable 96-well plates on standard laboratory equipment (see Figure 9). In such an assay, groups of hatched zebrafish embryos (48 hpf) were exposed to toxicants in the wells of 6-well plates in a 5-ml sample volume. Following 1–24-hour exposure, individual animals were transferred into the wells of a Luxcel plate (one animal per well in 10 uL of assay medium containing the oxygen probe), and the plate was sealed and analysed as described above.

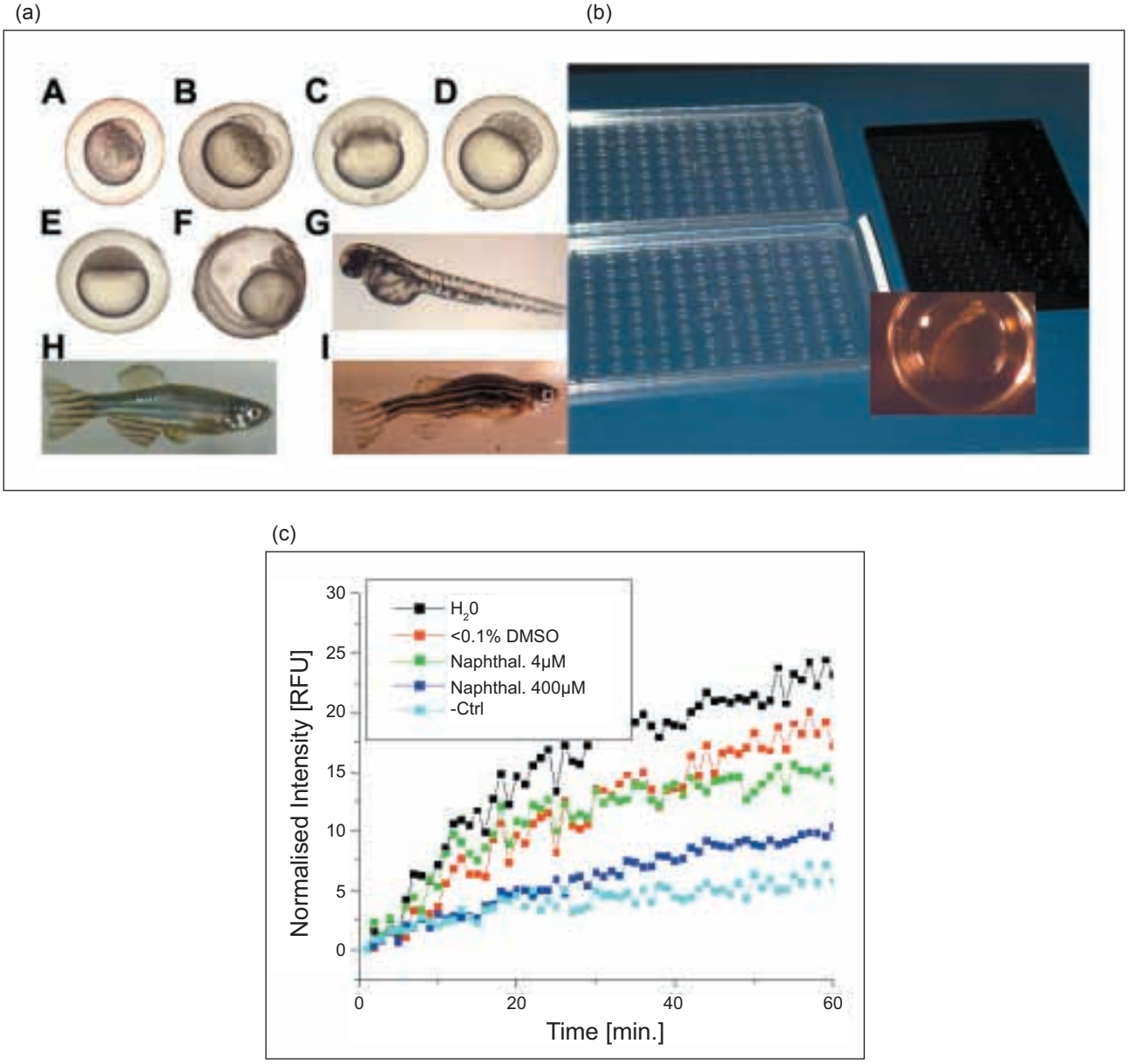


Figure 9: Different stages of development of *Danio rerio* (a) and images of Luxcel low-volume sealable 96-well plates (b). One assay well containing stage G zebrafish juvenile is magnified. Sample profiles (c) illustrate the effects of toxicant on organism respiration.

2.5 Toxicological Profiling using O_2 Respirometry with Panels of Model Organisms

A simple and versatile methodology for high throughput toxicological assessment of chemical and environmental samples was developed. It uses panels of test organisms ranging from prokaryotic (*E. coli, V. fischeri*) and eukaryotic (Jurkat) cells, to invertebrate (*Artemia salina*) and vertebrate (*Danio rerio*) organisms, to analyse alterations in their oxygen consumption by optical oxygen respirometry (Zitova et al., 2008). All the assays are carried out in a convenient microtitter plate format using commercial reagents (phosphorescent oxygen probe, microplates) and detection on a standard fluorescent plate reader, as shown in Figure 10. Simple experimental set-up and mix-and-measure procedure allow parallel assessment of up to 96 samples (or assay points) in 2 hours, and an easy generation of dose and time-dependent responses and EC_{50} values. The methodology was demonstrated with several different classes of chemicals including heavy metal ions, PAHs and pesticides, their mixtures and also validated with complex environmental samples such as wastewater from a WWTP. Representative toxicological data for different toxicants and their patterns of toxicity generated with the panel of organisms are shown in Table 5 and Figure 11.

This method has been shown to provide high sensitivity, sample throughput and information content, flexibility and general robustness. It allows ranking and profiling of samples, compares favourably with alternative methods such as MicroTox and mortality tests with animal models, and is well suited for large-scale monitoring programmes such as CWA and WFD.

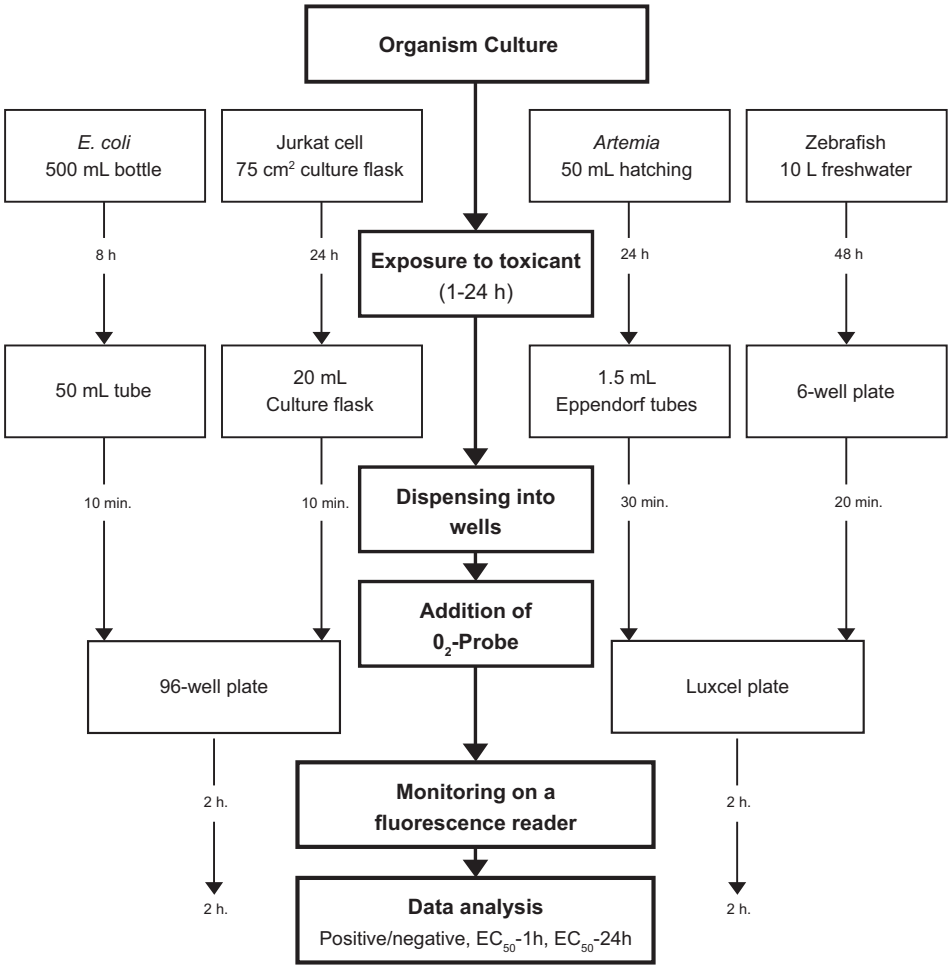


Figure 10: A flow chart for toxicological screening of environmental samples with a panel of model organisms, which shows the main operational steps and timing.

Table 5: A summary of toxicity data for different chemicals, model organisms and exposure times produced by the respirometric method and by conventional assays.

		Microtox 15 min	Respirometry 1 h	Respirometry 24 h
Organism	**Toxicant**	**EC_{50} [μM]**	**EC_{50}, ($C_{min.}$) [μM], [μM]**	**EC_{50}, ($C_{min.}$) [μM], [μM]**
V. fischeri	**Zinc**	38.2 [7]	528.4±34.7 (10)	–
	Copper	126.0 [7]	1145.0±50.3 (10)	–
	Lindane	165.0±34.4 [9]	–	–
	Pyrene	–	–	–
	Naphthalene	7.3 [50]	–	–
Artemia	**Zinc**	–	6756±437 (1000)	16.7±11.8 (10)
	Copper	–	823.2±150.0 (100)	45.0±16.3 (10)
	Lindane	–	ND (100)	31.6±25.2 (10)
	Pyrene	–	ND (1000)	9.1±4.6 (10)
	Naphthalene	–	ND (1000)	717.9±195.7 (100)
Zebrafish	**Zinc**	–	409.9±280.0 (100)	389.7±203.6, (100)
	Copper	–	656±384.2 (100)	20.2±11.2 (10)
	Lindane	–	252.2±212.0 (100)	79.8±57.0,(100)
	Pyrene	–	ND (1000)	157.0±62.1 (10)
	Naphthalene	–	ND (1000)	1098.5±139.5 (1000)
E. coli	**Zinc**	–	171.9±10.1 (100)	121.2±16.6 (10)
	Copper	–	473±7.9 (10)	469.1±106.2 (100)
	Lindane	–	ND (10)	ND (100)
	Pyrene	–	294.2±136.4 (10)	ND (1000)
	Naphthalene	–	ND (100)	ND (1000)
Jurkats	**Zinc**	–	278.6±50.6 (100)	147.2±28.3 (100)
	Copper	–	1234.0±54 (1000)	125.8±31.5 (100)
	Lindane	–	283.9±31.7 (10)	14.47±5.0 (10)
	Pyrene	–	195.7±90.6 (100)	37.52±4.49 (10)
	Naphthalene	–	1031±45.7 (1000)	516.6±128.3 (10)

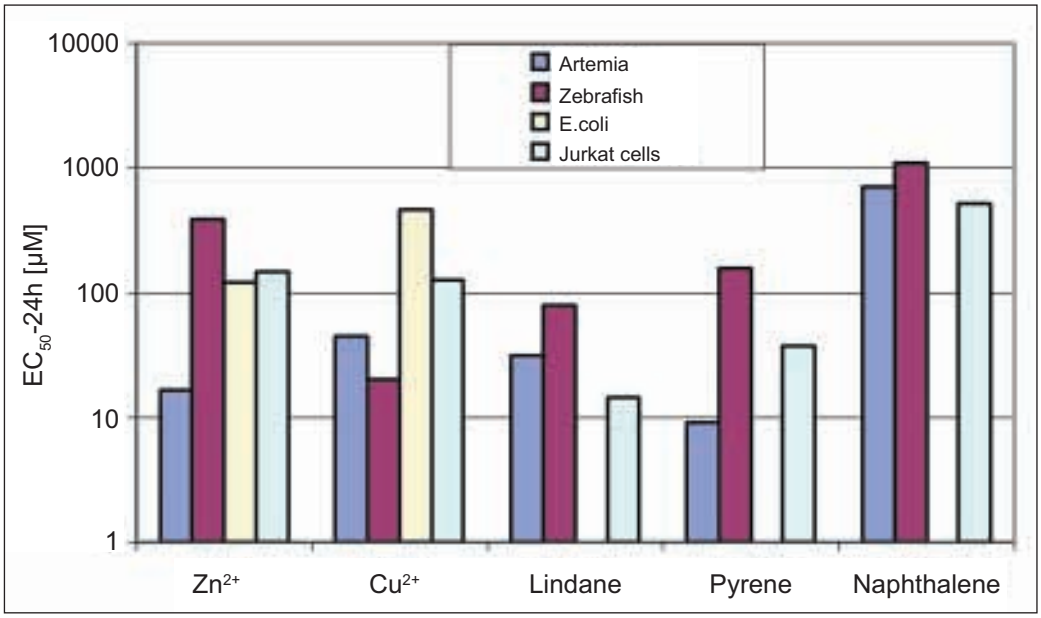


Figure 11: Graphical representation of toxicity patterns for different chemicals showing EC_{50}-24h values obtained with different test organisms and compared to each other.

2.6 Mechanistic Study of Toxicity of Microcystin-LR

Microcystins are potent environmental hepatotoxins – their main targets are cellular protein phosphatases PP1 and PPA2 and mitochondria, and their specificity is correlated with organic anion transporting peptides (OAPT). The effects of microcystin-LR (MCLR) on primary hepatocytes, HepG2 and Jurkat T cells and mitochondria from rat liver were analysed by measuring alterations in their oxygen consumption and other parameters of cellular function (Jasionek et al., 2008). MCLR was seen to inhibit oxygen consumption in primary hepatocytes with EC_{50} = 2.74 ± 0.65 nM, whereas HepG2 and Jurkat T cells showed no sensitivity to MCLR. An unusual uncoupling effect of MCLR on mitochondrial respiration was observed with glutamate/malate as a substrate, both in States 2 and 3. Facilitated delivery of MCLR into the cells by means of transfection reagents resulted in strong metabolic responses. A marked enhancement of respiration in HepG2 and inhibition of respiration in Jurkat T cells were observed, even at MCLR concentrations of 0.1–0.5 nM (see Figure 12). Cell viability, Adenosine Triphosphate (ATP) levels, extracellular acidification rate, reactive oxygen specie generation and intracellular phosphorylation were also measured and correlated with the respiratory responses. The data suggest that MCLR is a potent mitochondrial toxin and uncoupler of the electron transport chain (ETC).

Based on the results of this mechanistic study, a high-sensitivity screening assay for the detection of microcystins in environmental samples (contaminated water and algal blooms) was developed. This uses common cell line HepG2 and measurement of oxygen consumption. The assay provides a more convenient and ethical alternative to the conventional bioassays for microcystins based on primary cells isolated from animal tissue (rat liver).

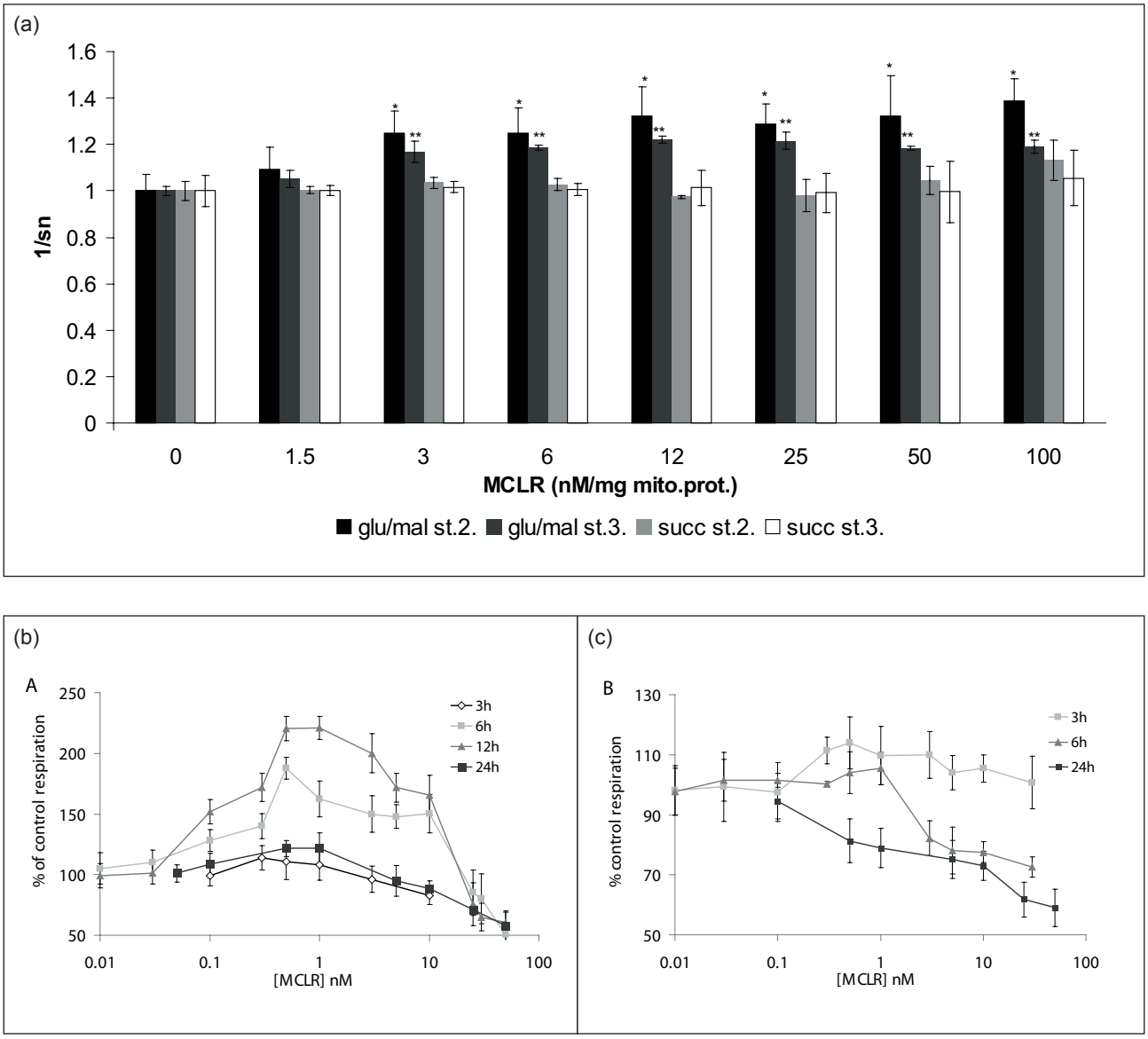


Figure 12: Uncoupling effects of MCLR on isolated rat liver mitochondria (a) measured glutamate/malate and succinate media and State 2 and State 2 respiration, respectively. Mitochondrial toxicity of MCLR in HepG2 (b) and Jurkat (c) cells measured with transfection reagent Endo-Porter at different exposure times (indicated).

2.7 Sensitive Enzymatic Assay for Neurotoxins

A new rapid, sensitive and high throughput screen for neurotoxicity of environmental samples (water) was developed. As shown in Figure 13, the assay is based on coupled choline oxidase/ cholinesterase system with acetylcholine and dissolved oxygen as substrates and the measurement of enzymatic oxygen consumption by optical oxygen sensing. In the presence of cholinesterase inhibitors O_2 consumption is suppressed, and the level of inhibition can be used for the quantification of neurotoxins in test samples. Two different platforms, namely the standard 96-well plate and fluorescent reader detection or glass capillary cuvettes and detection on LightCycler reader, were evaluated and compared for this assay. For each platform, assay development involved optimisation of working conditions in this bi-enzyme system, including enzyme and substrate concentrations, sample volume, temperature, timing, etc.

The assay was successfully applied to the detection of known cholinesterase inhibitors paraoxon and carbofuran and demonstrated good analytical performance and sensitivity. It is well suited for the purposes of environmental monitoring of water and marine samples contaminated with pesticides and other neurotoxins and cholinesterase inhibitors.

Principle scheme of enzymatic assay for cholinesterase inhibitors:

Cholinesterase

1) Acetylcholine $\rightarrow$ Choline + acetate - inhibited

Cholinoxidase

2) Choline + O_2 $\rightarrow$ Oxicholine - monitored

The team plans to continue work with this system, which will involve immobilisation of enzymes and the O_2-sensing probe on the microplates and microporous membranes. This approach is expected to provide further improvement in sensitivity together with a more convenient reagent-less assay format: simple addition of samples to assay wells and measurement. The assay needs to be validated with real samples, and corresponding partners are currently being sought.

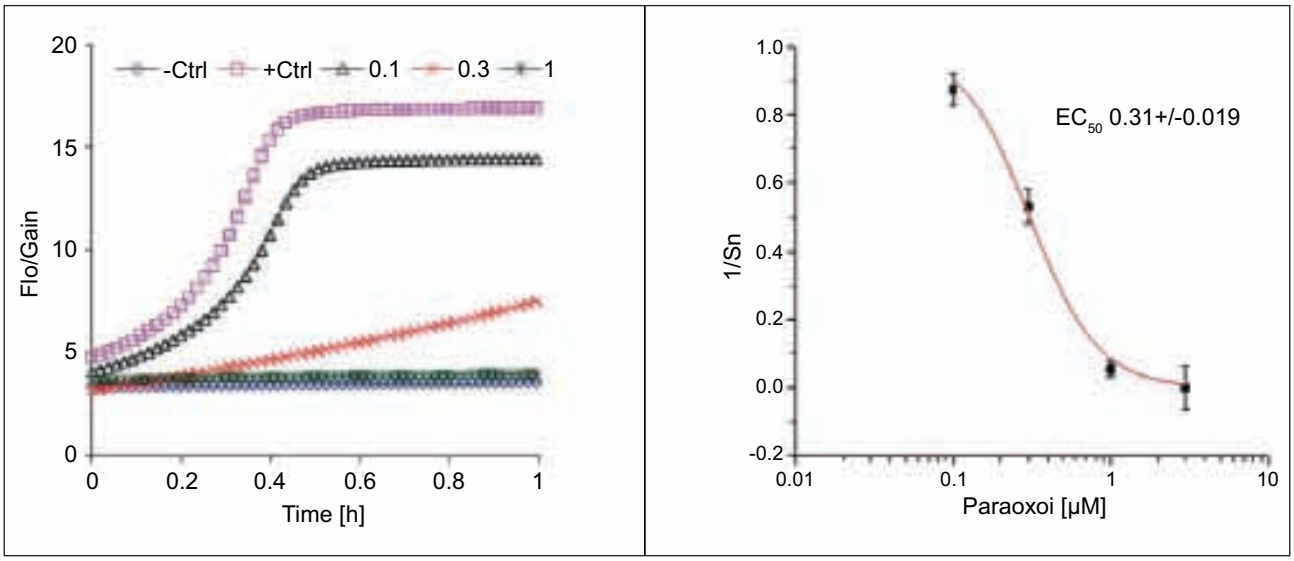


Figure 13: Principal scheme of the enzyme inhibition assay for neurotoxin, raw respirometric data and calibration curve for paraoxon measured in glass capillaries on the LightCycler reader (Roche).

2.8 Portable (Field) System for Respirometric Analyses

Based on the solid-state oxygen-sensitive materials and the phase-fluorometric detector previously developed by the project team (Papkovsky, 2004), a prototype of a portable system for respirometric analyses has been designed (see Figure 14). This operates with small glass vials to which test samples are placed and then measured non-invasively through the bottom of the vial with the fibre-optic probe of the detector. Due to the other more important tasks conducted (see above) and also time constraints and logistics of the work, this system was only briefly evaluated in this project. However, it is now being used actively and successfully in a separate satellite project on the environmental monitoring of chemicals, which is also funded by the EPA (doctoral scholarship). The results generated from this system will be presented in a separate report (when work is completed).

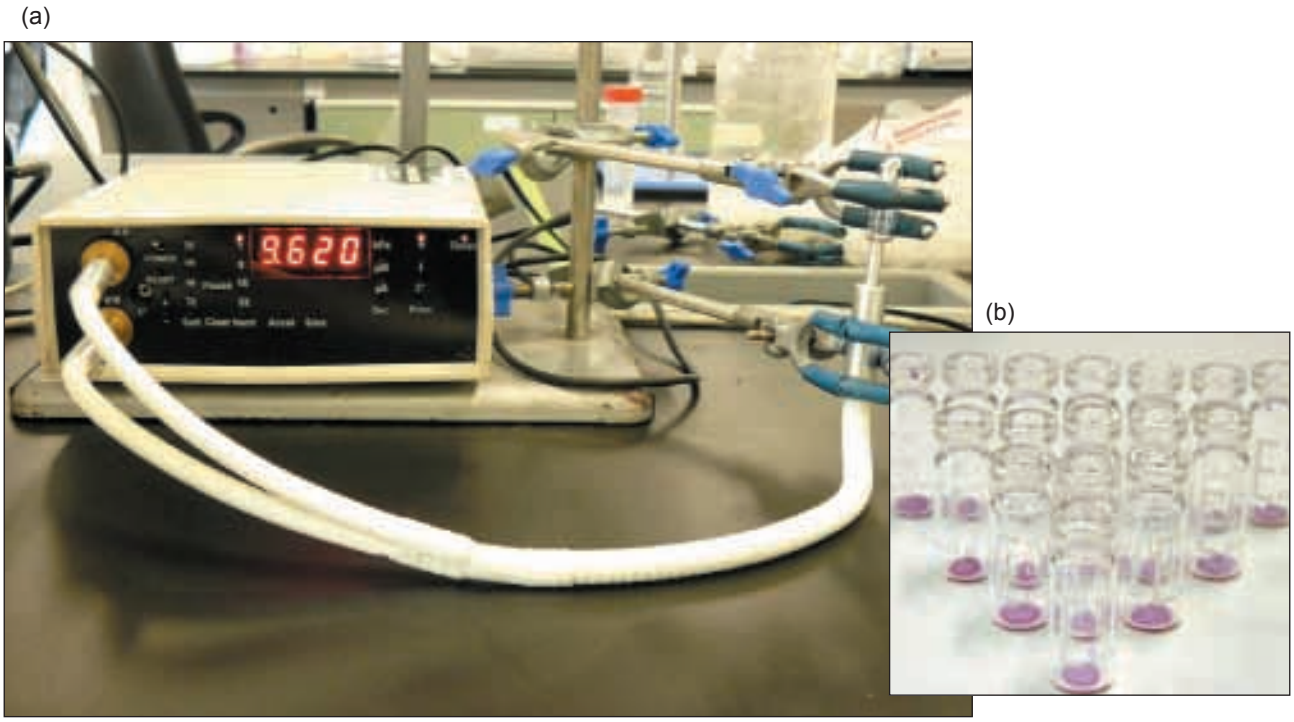


Figure 14: Measurement set-up of the portable respirometric system (a) and test vials used with it (b).

2.9 Analysis of Intracellular Oxygen and Metabolic Responses of Mammalian Cells by Time-resolved Fluorometry

A simple, minimally invasive methodology for the analysis of intracellular oxygen in populations of live mammalian cells was developed (O'Riordan et al., 2007). Loading of the cells with the phosphorescent O_2-sensing probe, MitoXpress (Luxcel Biosciences) was achieved by passive liposomal transfer or facilitated endocytosis in standard microwell plates, followed by monitoring on a time-resolved fluorescent reader. Phosphorescence lifetime measurements provided an accurate, real-time, quantitative assessment of local oxygen concentration within the cells, and changes in cellular oxygen levels in response to stimulation. Analytical performance of the method was examined and optimised, and then demonstrated with different suspension and adherent cell lines including Jurkat, PC12, A549, HeLa, SH-SY5Y and C2C12. Characteristic responses to mitochondrial uncouplers, inhibitors, plasma membrane depolarisation and Ca^{2+} effectors were monitored and correlated with literature data on the mechanisms of action of these effectors. The representative profiles are shown in Figure 15.

This methodology has already been applied in a number of mechanistic and metabolic studies with different models and disease states (Zhdanov et al., 2008). It is particularly useful for toxicological studies, allowing sensitive detection of minor, sub-lethal injuries of the cells, monitoring of small, rapid and transient changes in cell respiration and screening of new chemical entities and environmental samples. The new assay provides relevant, information-rich data on cellular function and metabolism.

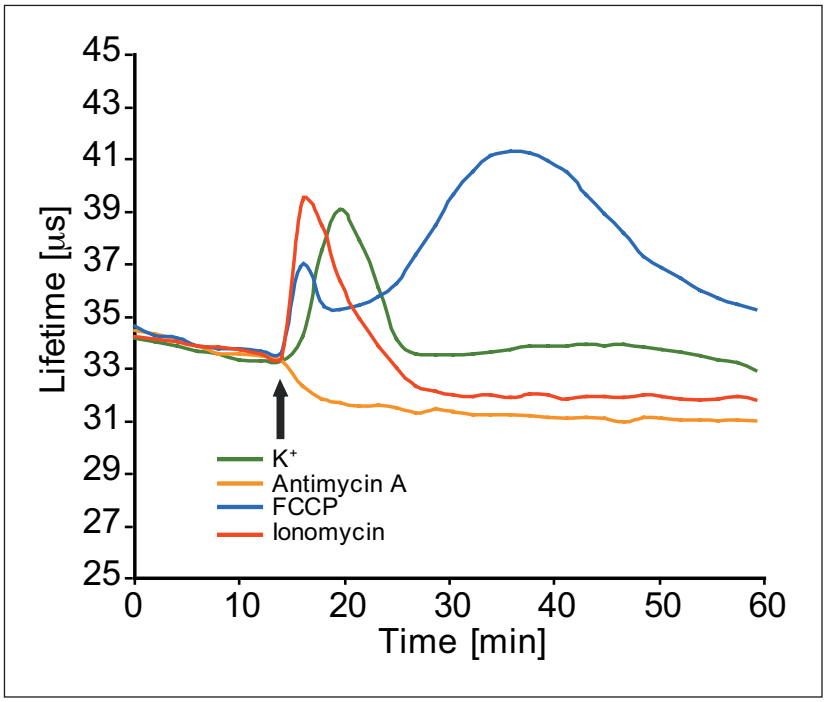


Figure 15: Phosphorescence lifetime profiles of neurosecretory PC12 cells loaded with MitoXpress probe and treated with: 100 mM KCL (green), 4 µM antimycin A (yellow), 4 µM FCCP (blue) and 1 µM ionomycin (red). Measurements were conducted on the Victor2 reader at 37 °C.

3 Validation and Dissemination

The new toxicological screening systems and applications were applied to the analysis of the following environmental, industrial samples (taken from real-life environment):

- Water samples from wastewater treatment plants – total toxicity.
- Industrial effluents – total toxicity.
- Food samples – total microbial load.
- Water samples contaminated with algal blooms (microcystins).

The team conducted joint studies (mini-projects) with various toxicants, environmental and food samples with the following partners: EPA Laboratory in Inniscarra, Co. Cork; the Marine Institute Aquatic Toxicology laboratory, Shannon, Co. Clare; Campden-Chorleywood Food Research Centre, Campden, United Kingdom; Microchem, Dungarvan, Co. Waterford; the Research Centre for Environmental Chemistry and Ecotoxicology, The Czech Republic (ongoing). The possibility of running a small-scale pilot trial on the analysis of water samples from the Western water basin has also been discussed with the Marine Institute and the EPA. In addition, a number of scientific contacts with European environmental research organisations and monitoring laboratories have been established.

Through the activities of our industrial partner Luxcel Biosciences, the project team became involved in collaborations and discussions with a number of companies and academic institutions, including the two Irish companies Glanbia and Microchem who became interested in evaluating the rapid microbial and sterility tests developed by the team. Two batches of food samples were provided to the team's laboratories and analysed for microbial contamination. Luxcel Biosciences has initiated the Association of Analytical Communities (AOAC) certification of the total aerobic viable counts test for food samples (filed in May 2008).

The team participated in the following workshops:

1 1st Water Sensor Programme Annual Workshop organised by the Marine Institute and the EPA, March 2006, Dublin. Presentations by Prof. D. Papkovsky and by Prof. J. Davenport.

2 Science Foundation of Ireland-National Institute of Health USA International Workshop on Sensors, February 2007, Dublin. Prof. D. Papkovsky: Phosphorescence based sensors.

3 2nd Water Sensor Programme Annual Workshop organised by the Marine Institute and the EPA, March 2007, Oranmore, Galway. Presentations by Prof. D. Papkovsky and Dr R. Fernandes.

4 EPA Water Research Workshop, October 2006, Carlow. Attended by Prof. D. Papkovsky.

4 Project Outcomes

A significant number of publications in scientific literature was generated based on the results of project work, including primary papers, book chapters, conferences abstracts and presentations. A new Patent Cooperation Treaty application has been filed in June 2008, to protect the new method of toxicological assessment. The details of these are given below.

4.1 Book Chapters

1 Zitova A., O'Mahony F.C., Cross M., Davenport J., Papkovsky D.B. Biological toxicity testing of heavy metals and environmental samples using fluorescence based oxygen sensing and respirometry. In: ***Advanced Environmental Monitoring***, Y.J. Kim and U. Platt (eds.), Springer 2007, Ch.24.

4.2 Original Papers in Peer-reviewed Journals

1 O'Mahony F.C., Papkovsky D.B. Rapid high-throughput assessment of aerobic bacteria in complex samples by fluorescence-based oxygen respirometry. ***Appl Environ Microbiol***. 2006 Feb.; 72(2): 1279–87.

2 Ogurtsov V.I., Hynes J., Will Y., Papkovsky D.B. Data analysis algorithm for high throughput enzymatic oxygen consumption assays based on quenched-fluorescence detection. ***Sensors and Actuators B: Chemical***, B, 2008, 129(2): 581–90.

3 O'Riordan T.C., Zhdanov A.V., Ponomarev G.V., Papkovsky D.B. Analysis of intracellular oxygen and metabolic responses of mammalian cells by time-resolved fluorometry. ***Analytical Chemistry***, 2007 Dec. 15; 79(24): 9414–9.

4 O'Mahony F.C., Green R.A., Baylis C., Fernandes R., Papkovsky D.B. Analysis of total aerobic viable counts in samples of raw meat using fluorescence-based probe and oxygen consumption assay. ***Food Control***, 2008, DOI: 10.1016/j.foodcont.2008.03.003

5 Zitova A., O'Mahony F.C., Cross M., Davenport J., Papkovsky D.B. Toxicological profiling of chemical and environmental samples using panels of test organisms and optical oxygen respirometry. ***Environ. Toxicol***, 2008 (in press).

6 Schouest K., Zitova A., Spillane C., Papkovsky D.B., Toxicological assessment of chemicals using Caenorhabditis elegans and optical oxygen respirometry. ***Environ. Toxicol. Chem***., 2008 (in press).

7 Zitova A., Cross M., Hernan R., Davenport J., Papkovsky D.B. Respirometric acute toxicity screening assay using *Daphnia magna*. ***Environ. Toxicol***, 2008 (submitted).

8 Jasionek G., Zhdanov A.V., Davenport J., Papkovsky D.B., Investigation of mitochondrial toxicity of microcystin-LR. ***In Vitro Toxicol***., 2008 (submitted).

4.3 Conference Presentations

1 Papkovsky D.B., Sensing cellular respiration: new methods and practical uses, Eur. Conf. Optical Chemical Sensors and Biosensors, April 2006, Tuebingen, Germany.

2 Papkovsky D.B., O'Riordan T.C., Fluorescence based sensing of cellular respiration, 9th World Biosensors Congress, May 2006, Toronto, Canada.

3 Papkovsky D.B., Biological toxicity testing using optical oxygen sensing and respirometry. 6th International Symposium on Advanced Environmental Monitoring, 27–30 June, 2006, Heidelberg.

4 O'Mahony F.C., Papkovsky D.B. Rapid high throughput assessment of aerobic bacteria in complex samples by fluorescence-based oxygen respirometry. 2nd Federation of European Microbiological Society Congress of European Microbiologists, 4–8 July 2006, Madrid.

5 O'Mahony F.C., Zitova A., Cross M., Davenport J., Papkovsky D.B. Biological toxicity testing of chemical and environmental samples using optical oxygen sensing and respirometry. Analytical Research Forum, 17–19 July 2006, University College Cork, Ireland.

6 O'Mahony F.C., Biological toxicity testing of chemical and environmental samples using optical oxygen sensing and respirometry. Environmental Forensics: Chemical, Physical and Biological Methods, 18–21 September 2006, University of Durham, United Kingdom.

7 Papkovsky D.B., New toxicological screening systems, 'Nano2Life' Conference September 2006, 6th Framework Programme of the EU Network, Cork.

8 Zitova A., O'Mahony F.C., Cross M., Davenport J., Papkovsky D.B., Toxicological profiling of chemical and environmental samples using panels of test organisms and optical oxygen respirometry, Monitoring and assessment of river pollutants, May 2007, Lisbon, Portugal.

9 Papkovsky D.B., Analysis of enzyme activity by quenched-fluorescence oxygen sensing, International conference Biocatalysis-2007, July 2007, Moscow, Russia.

10 Papkovsky D.B., Screening for mitochondrial toxicity and metabolic responses of cells using phosphorescent O_2-sensing probes, 1st Internat. Congress on Drug Discovery and Development, February 2008, Dubai.

11 Papkovsky D.B., O'Riordan T.C., Zhdanov A., Phosphorescent probes for the analysis of intracellular oxygen and real-time monitoring of cell respiratory responses, Keystone Symposium on Hypoxia, February 2008, Vancouver.

12 Zitova A., Jasionek G., Papkovsky D.B., Toxicological assessment of chemicals and marine toxins base on optical oxygen screening, Society of Environmental Toxicology and Chemistry Conference, May 2008, Warsaw.

13 Zhdanov A.V., Papkovsky D.B., Analysis of respiratory responses of neuronal cells to the decrease of extracellular calcium, European Bioenergetics Congress 2008, July 2008, Dublin.

14 Papkovsky D.B., Ponomarev G.V., Sensing and imaging of (intra)cellular oxygen by means of the phosphorescent porphyrin probes, 5th Internat. Conf. on Porphyrins and Phthallocyanines, July 2008, Moscow.

4.4 Patent Applications

1 Papkovsky D.B., Jasionek G., Zhdanov A.V., Method of toxicological assessment, PCT Application filed by University College Cork on 26 June, 2008.

The above publications emanated directly from project work. Many of them describe new approaches to environmental monitoring, new technical solutions and scientific findings obtained using these tools and bioanalytical systems. The results were peer-reviewed and published in high-profile environmental and bioanalytical journals. They provide a substantial contribution to the area of marine and environmental research and monitoring, producing a marked impact on the community and industry.

5 Discussion

The above summary of experimental data generated in the course of the project, together with the more detailed description and discussion of the results in the corresponding papers (eight journal articles have been published or submitted so far), demonstrate that the new technology of optical oxygen sensing and respirometry provides a useful tool for environmental monitoring and biological testing of toxicity of various chemical and environmental samples.

A significant development of this technology, its application to various biological models, chemical and environmental samples and extensive validation of the panel of new toxicity assays were undertaken under this project. In particular, respirometric assays have been established for several new, very useful cell and animal models which have not previously been examined in such applications. These animal models included, for example, *Daphnia magna* which is regarded as one of the golden standards in traditional toxicity testing of chemical and water samples. Currently, rather simple but at the same time time-consuming and not very objective mortality/immobilisation based assays are used with *Daphnia* (also some other animals), which are prone to false-positive results and have limited scalability, whereas the new respirometric assays have provided automation, increased sample throughput and general convenience. In many cases, they also provide higher sensitivity to the toxicants which is understandable as they are based on the monitoring of altered metabolism and respiration of test animals which normally occurs prior to their death. This allows the detection of sublethal toxic effects which cannot be picked by conventional tests. Several other animal models, particularly *Danio rerio* (zebrafish) embryos and juveniles, *Artemia salina, C. elegans*, have been used successfully. These organisms are currently actively used for the analysis of gene and protein function, various disease states and drug development. They are therefore of particular relevance to biochemical toxicology and environmental monitoring.

Respirometric toxicity assays have also been used in conjunction with mammalian cell lines (Jurkats, PC12, HepG2, HeLa), and also with microbial cells. Moreover, the aforementioned models and respirometric assays were bundled together to provide panels of test organisms ranging from prokaryotic and eukaryotic cells to invertebrate and vertebrate organisms, to conduct more detailed toxicological assessment of chemical and environmental samples. These panels of organisms and respirometric assays allow profiling of different toxicants on the basis of their toxic action on different models. This method also facilitates predictive identification of toxicants in unknown samples and analysis of complex mixtures.

Throughout the project, the basic water-soluble oxygen probe MitoXpress has been used in the majority of the assays, where it has demonstrated excellent performance, stability and suitability for all the models and classes of toxicants used in this study. This probe has been used in conjunction with the different formats of optical oxygen respirometry, including standard 96- and 384-well microtitter plates, the low-volume sealable 96-well plates (Luxcel) and the glass capillary microcuvettes (Roche). It has been measured on several commercial instruments that implement different detection modes, including conventional steady-state fluorescence (SpectraMax Gemini reader from Molecular Devices, LightCycler from Roche, Genios from Tecan), microsecond time-resolved fluorescence (Victor reader from PerkinElmer, Genios from Tecan, ArcDia from Luxcel) and phosphorescence lifetime measurements (Victor from PerkinElmer, ArcDia from Luxcel). When used at working concentrations (100 nM–1 µM), the probe was easily detectable on all these instruments, and it demonstrated high sensitivity and selectivity and stability to interferences by sample components and toxicants. The TR-F and lifetime based

detection proved to be advantageous for the sensing of dissolved oxygen concentration and oxygen respirometry applications.

In parallel with the main (high-throughput) platform, which uses a water-soluble O_2 probe and fluorescent plate reader detection, a second, portable platform based on the solid-state O_2-sensors and phosphorescence phase detector was developed and evaluated in respirometric toxicity assays. This approach also proved viable, the second platform is deemed particularly suited for conducting biological toxicity assays with small panels of fully sealed samples, and also for field use.

At the final stages of the project, high practical potential of the optical oxygen sensing and respirometry methodology was demonstrated by conducting several detailed mechanistic and toxicological studies and new, enabling developments to this technology. This included the elaboration of the new mechanisms of toxicity of microcystins – important marine toxins from algal blooms (see[10], the development of new method for sensitive detection of MCLR with alternative (non-primary) cell models (see patent application), the development of a new method of measurement and real-time monitoring of intracellular oxygen concentration in mammalian cells.

Overall, the new respirometric assay platform and corresponding chemistries, accessories and standardised procedures developed under this project provide new versatile tools for researchers and a viable alternative to existing biological toxicity testing. They gradually gain their momentum and broader use in biological, toxicological and environmental laboratories, in Ireland and abroad. These techniques and assays complement well the existing techniques and provide a broad scope for their further development and application.

6 Conclusions

6.1 Overall Conclusions

The overall conclusion is that the project has succeeded in achieving its aims and the practical outcomes that were expected from this research programme and the funding provided. Moreover, a number of additional accomplishments have been made, including the rapid microbial assay in food and environmental samples, the toxicological profiling platform, the extended panel of model organisms (C. *elegans*, various prokaryotic and eukaryotic cell lines), and the intracellular oxygen sensing assay. The new respirometric platform and a panel of biological toxicity assays and screening systems have been developed. A range of different biological models have been evaluated. These assays have been successfully validated with sets of real samples, benchmarked against the established toxicity tests. By the end of the project a number of these respirometric systems have been brought to a stage where they can be deployed in a variety of high-utility applications and analytical tasks, including toxicological and environmental screening and monitoring.

6.2 Specific Conclusions

1 Respirometric toxicity assays have been developed for the following test organisms: *Daphnia magna, Artemia salina* and *C. elegans* (invertebrates), zebrafish embryos (vertebrate), HL60, HepG2 and Jurkat T cells (eukaryotes), *E. coli, P. fluorescence* and *V. fisheri* (prokaryotes).

2 Using these model organisms, a number of representative toxicants of different type were assessed, including heavy metal ions (Cd^{2+}, Zn^{2+}, Co^{2+}), chemicals ($K_2Cr_2O_7$, phenol, sodium lauryl sulphate), PAHs, pesticides, marine and food toxins, drugs and pharmaceuticals.

3 Several batches of wastewater samples collected by the local EPA laboratory from water-treatment plants in the Cork area were screened using the above screens and test organisms and toxicity data were generated. A panel of contaminated wastewater samples was received and examined jointly with Shannon Aquatic Toxicology Laboratory. A study is ongoing with a panel of water samples contaminated with microcystins received from The Czech Republic.

4 The new respirometric assays were benchmarked against the established acute toxicity assays currently widely used in environmental and toxicological laboratories, including the Microtox® system (*V. fisheri*), the *Daphnia* test, conventional cell viability assays and other markers of toxicity. In the majority of cases the respirometric toxicity assays and measurement approach showed comparable or better sensitivity, the ability to detect sub-lethal effects and doses of toxicants, and clear advantages in assay throughput, automation, miniaturisation, general convenience and robustness.

5 Using panels of these test organisms, patterns of toxicity were obtained for a number of toxicants reflecting the dose, time and test organism dependence of their toxicity. These were seen to be characteristic to the particular toxicant and its mode of toxic action. This approach potentially allows identification of toxicants or particular type of contamination in unknown samples such as environmental samples or industrial waste.

6 Using the high throughput capabilities of the optical oxygen respirometry and the above test organisms (individually or in combination), experiments with mixtures of toxicants were conducted, aiming at evaluating their combinatorial effects and biological hazard.

7 Rapid test for total load of aerobic bacteria and/or sterility has been developed and applied to different microorganisms. The test was applied to the enumeration of total viable counts in food samples (raw meat, swabs), and it was successfully validated by the food industry as a more simple, rapid and cost-effective alternative to the conventional total viable counts (TVC) test on agar plates.

8 A detailed study on the mechanisms of toxicity of MCLR on different model organisms has been conducted, particularly measuring respiration and other biomarkers in cultured cell lines, primary hepatocytes from rat liver and isolated mitochondria. A new high-sensitivity cell-based assay for the presence of microcystins in water samples was developed which has the potential for environmental screening and monitoring.

9 A new toxicity assay for neurotoxins (cholinesterase inhibitors) was developed using the bi-enzymatic reaction (cholinesterase-choline oxidase) coupled with optical O_2 detection. The system currently provides sub-nM sensitivity and has the potential for further improvement. It looks attractive for environmental monitoring (phosphororganic pesticides), and complements well animal-based testing. The team has plans to continue further development of this system and validation with real samples.

10 Integration of these high throughput toxicity assays based on optical oxygen respirometry into one screening system has been achieved which makes it compatible with all the above toxicity assays and applications.

11 Initial development of a portable, low-to-medium throughput version of the respirometric toxicity testing system(s) for field use was conducted. Further R&D work in this direction is necessary.

12 Results provided the basis for a significant number of journal publications, conference presentations, environmental workshops, and one patent application. This has further improved the team's high international standing and its leading positions in the area, both in research and practical applications.

6.3 Recommendations and Future Work

Further concerted actions by the Marine Institute, the EPA and the project team towards commercialisation, broader dissemination and deployment of this new, emerging environmental technology are deemed necessary. This will facilitate the harvesting of the results and the extraction of maximum value from this project. Investment in further R&D in this technology, which has been developed and implemented in Ireland and which addresses current needs of environmental monitoring, is expected to have a significant impact and lead to new discoveries and monitoring systems. A number of potential avenues and spin-off projects emanating from this project have been identified by the team.

In particular, the development and biological application of intracellular O_2 sensing technology, which has proven its high utility for many areas of life science, is one of the top priorities for the team. Recently, project co-ordinator Prof. D. Papkovsky has secured a sizeable Investigator Programme grant from the Science Foundation of Ireland – over €1.1M in total over four years, starting from September 2008, which is directed to continue basic development of this technology for use in general cell biology and analysis of cellular function (targeted O_2 probes, mapping O_2 in cells/tissues, hypoxia, signalling pathways in cells). However, it is important that this support is augmented by the funding for applied science, and particularly for new practical uses of this technology in environmental monitoring and toxicology.

In addition, several new applications of optical oxygen sensing identified in the current project and demonstrated as proof of concept pave the way for new R&D projects. Possible projects, which are considered to be within the scope of interests and funding programmes of the EPA and Marine Institute (ongoing or in planning states), include, for example:

- Development of systems for multi-parametric assessment of impaired cellular function caused by environmental samples and toxins, with O_2 sensing/respirometry assays as one of the main components which is combined with a panel of other important biomarkers.

- Screening of libraries of compounds/extracts derived from marine or natural sources, early-state assessment of their mitochondrial toxicity and safety, and potential drug candidates.
- Investigation of common hepato and neurotoxins (e.g. from cyano-bacteria, algal blooms, or other sources) by optical oxygen respirometry: mechanisms of toxicity, sensitive detection.
- Toxicological assessment of new nanomaterials and nanoparticles of industrial origin by optical oxygen respirometry: mitochondrial toxicity, interference with cellular function, mechanistic studies.
- Selective determination of food-born pathogens (coliforms, Salmonella, Lysteria, Campylobacter, other bacteria) in food and environmental samples by optical oxygen respirometry.
- Analysis of pesticides and cholinesterase inhibitors based on immobilised oxygen dependent enzymes or coupled enzymatic systems.

The project team plans to apply for R&D funding for these and for some other projects. Relevant programmes and initiatives are currently being sought in Ireland and abroad, however, targeted funding from the Marine Institute and the EPA is highly desirable to keep momentum, retain and further strengthen the research team, expertise and critical mass.

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Abbreviations and Acronyms

CWA	United States Clean Water Act
EC_{50}	effective concentration causing 50% of the effect
EPA	Environmental Protection Agency
ETC	electron transport chain
EU	European Union
FCCP	Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
MCLR	microcystin-LR (hepatotoxin from algal blooms)
O_2	molecular oxygen
OAPT	organic anion transporting peptides
PAHs	polyaromatic hydrocarbons
PP1, PPA2	protein phosphatases 1 and A2, respectively
SLS	sodium lauryl sulphate
TVC	total viable counts (microbial contamination)
WFD	EU Water Framework Directive
WWTP	wastewater treatment plants

An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil agus Rialtais Áitiúil a dhéanann urraíocht uirthi.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail.
- Scardadh dramhuisce

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

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