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Marine Institute
Foras na Mara

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Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

STRIVE
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# Demonstration of a Miniaturised Multi-Channel Cytometry System and Its Suitability for Autonomous Deployment

SSTI
Strategy for Science, Technology and Innovation

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

# Environmental Protection Agency

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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- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet several times a year to discuss issues of concern and offer advice to the Board.

**Marine Institute/Environmental Protection Agency Partnership: Advanced Technologies for Monitoring Water Quality**

**STRIVE Programme 2007–2013**

# Demonstration of a Miniaturised Multi-Channel Cytometry System and its Suitability for Autonomous Deployment

## (AT-04-01-07)

## STRIVE Report

Prepared for the Marine Institute and the Environmental Protection Agency

by

Tyndall National Institute, University College Cork

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## DISCLAIMER



The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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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 implementation of the Water Framework Directive
- To provide technological support for the sustainable development of aquatic/marine resources, and
- To 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 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 the development of innovative solutions to important environmental questions. The SmartBay project is advancing with the input of a wide range of agencies, researchers, industry and end-users.

The EPA and the 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 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 (ETAP).

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, the expertise and capability developed from the agencies' shared investment.

In the current economic climate, co-operation 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 co-operation and is a vital support in the development of Ireland's Smart Green Economy. This co-operation 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
EPA

**Dr Peter Heffernan**
Chief Executive
Marine Institute

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# Executive Summary

## 1 Background

Water quality monitoring is recognised as an important issue for public health. Current approaches typically involve sample collection and transport for analysis at a central facility. Methods of detection for bacteria such as *Escherichia coli* involve the use of microbiological or immunological techniques that are usually laboratory based, time consuming and performed by highly trained personnel. Flow cytometry is a high-tech alternative for the counting and identification of cellular species, offering faster, automated analyses and multi-parameter information. Flow cytometry is not routinely used for water quality monitoring, primarily due to the associated costs and the complexity of instrument operation. The majority of conventional systems are not portable due to the optical set-ups employed.

## 2 Specific Objectives of the Research

- Design, fabrication and testing of a lab-based demonstration/proof-of-principle cellular analysis unit showing the required sensitivity of the proposed autonomous system. Incorporation of flexible autonomous hardware with programmable volumes and timings between samples, a pre-filter/cell capture chamber, microfluidics for labelling/marker addition and cytometry channels and fluorescent counting.

- Selection and implementation of immunological techniques for identification of cellular species of interest, e.g. *E. coli*, with subsequent benchmarking against standard reference methods.

- Assessment of potential risks to system operation such as clogging and antifouling, including trials of operation and cleaning protocols using fresh and marine samples with varying degrees of particulate loading.

## 3 Results

While progress towards the development of an alignment-free optofluidic system for cytometry was demonstrated, a fully functioning prototype capable of performing cell counts was not realised. Partial integration of highly sensitive, space and power-efficient components (avalanche photodiodes and microLEDs) onto a single platform was achieved using specialist flip-chip alignment and attachment methods. Subsequent integration with microfluidic channels was performed but the detection of individual particles was not resolved from background noise. While particle counts were not achieved, a functioning detector array was demonstrated which would be applicable for particle counting and other optofluidic analysis applications. Outputs of the research programme also include the design of innovative approaches to the heterogeneous integration of key optical elements which provides the foundations for the demonstration of the system concept. The preferred embodiment of the optical module design includes a light source and detection element pre-aligned to a microfluidic channel without the need for laser processing of detector modules, which was a key issue throughout the project. Additional funding is currently being sought to allow further development of the system. The acquisition of new specialist instrumentation for the advanced packaging facility in the Tyndall National Institute offers new solutions to issues encountered during the work programme.

The development of an immuno-labelling protocol for *E. coli* detection was explored for molecular-level biological recognition. Bench-top flow cytometry and conventional immunofluorescent microscopy were utilised for the enumeration of antibody-tagged *E. coli* in water samples. Optimisation was carried out using two commercially available antibodies but no statistical difference in staining proficiency was found. The percentage staining was relatively low for both antibodies at approximately 10%. This demonstrates that more specific antibodies are required for the detection of this bacterium. Of the two immunological methods utilised, flow cytometry was found to be the most appropriate for the detection of specific bacteria in the environmental samples tested.

The magnitude of clogging at the microfluidic scale with the flow rates envisaged for cytometer function was investigated. The results of the initial flow trials performed over several hours showed no clogging by natural water or pure cell culture samples, even at high particle concentrations ($<3 \times 10^6$ cells/ml). An assessment of the fouling performance of typical fabrication materials remotely deployed under different conditions of flow and fouling pressure suggests that all could be incorporated in a flow cytometer design without significant advantage/disadvantage. Results to date indicate that the proposed cytometer design should not be prone to frequent blockages, or require pre-filtering.

# 4 Conclusions and Recommendations

- A fully functioning prototype cytometry system has yet to be demonstrated.

- Innovative approaches to the development of convergent technology platforms, including the on-chip integration of high sensitivity miniaturised optics with fluidic networks, have been explored.

- The greatly reduced size and potential low cost of the core optofluidic technology developed during the project facilitate integration into the next generation of miniaturised and hand-held portable sensing devices.

- Key developments needed include the tailored design of optical components with added functionality, such as wavelength-specific detection capability.

- Consideration of system components and interactions is needed at design stage to facilitate broader access to alignment-free diagnostic platforms.

- Direct antibody detection in conjunction with flow cytometry could eventually be used in addition to, or replace, the standard microbiological methods for detection and enumeration. Additional development and optimisation of new antibodies are required.

- Clogging trials need to be pursued over longer periods to monitor the development of biofilms within fluidic channels. The incorporation of internal flushing procedures or the use of antifouling coatings on external surfaces of deployed sensors also needs to be considered.

- Flow cytometry-based technologies have immense global market potential as highlighted in a recent Frost and Sullivan Report[1]. Preliminary market findings suggest that the clinical diagnostic application domain is a leading-edge market; however, significant issues in relation to sample preparation must be overcome. Subject to the further application-specific development described above, it is believed that a take-up in the clinical domain would follow demonstration of the environmental unit.

- Two Irish-based start-up companies have expressed interest in the commercial exploitation of the outputs of this research. Researchers have also interacted closely with SensL, a spin-off company from the Tyndall National Institute, on the development of the detection array.

- Current and future outputs of this research have significant potential to positively impact the Irish economy through the development of innovative technologies for environmental assessment, application-driven development in conjunction with technology start-ups, and the exploitation of transfer and licensing opportunities.

1. US Flow Cytometry Markets. Frost & Sullivan, March 5, 2008. 87 pp. Pub ID: MC1696543

# 1 Introduction

## 1.1 Overall Objectives

The presence of certain micro-organisms, including *Escherichia coli* and toxic algae, in Irish marine and freshwater environments poses a continuing concern for public health. Routine identification of these species typically involves sample transport to a specialist laboratory for labour-intensive characterisation and enumeration by highly trained personnel. Techniques such as microscopy and bacterial culture are routinely employed, with identification of *E. coli* achieved following filtration and 24-h incubation on selective media.

Once released to surface and marine waters, it has been suggested that, while *E. coli* maintains its virulence, its colony-forming ability is affected, which could result in an underestimation of total viable counts by traditional culture techniques (Schwartz-Mittlemann *et al.*, 2002). The incubation time needed for bacterial culture (24 h or more) also leads to a significant delay in reporting a potential pollution event. Faster analysis can be achieved using kits such as IDEXX Colilert®, but incubation times of several hours are still required.

Flow cytometry can be used as an alternative method for the detection of single-celled organisms including phytoplankton (Marie *et al.*, 2005). Advantages of this technology include the high speed of analysis (taking minutes as opposed to hours), the multi-parameter information that can be obtained in a single run, including cell size, cell diameter, viability, etc., and the ability to automate the analysis. Issues with the use of current commercial flow cytometry systems include the high cost of purchase and maintenance, the need for skilled operators to align optics and calibrate the system, and the relatively large area of dedicated bench space these systems require. In addition, most commercial systems consume significant volumes of sheath fluids and are not portable or amenable to use outside of the laboratory. Miniaturisation of conventional instrumentation would ideally allow the development of low-cost, turnkey systems exhibiting a high degree of process integration, automation and portability.

## 1.2 Specific Objectives of the Research

The overall aim of the Tyndall–Department of Zoology, Ecology and Plant Science (ZEPS) University College Cork (UCC) cytometry collaboration is the provision of new technology-based monitoring solutions for environmental analysis – the development of a relatively low-cost high-throughput system for cell monitoring.

The specific objectives of the research programme were:

- Design, fabrication and testing of a lab-based demonstration/proof-of-principle cellular analysis unit showing the required sensitivity of the proposed autonomous system. The use of miniaturised, power-efficient and highly sensitive optical components was investigated to allow the development of rugged systems more suited to field analysis. Incorporation of flexible autonomous hardware with programmable volumes and timings between samples, a pre-filter/cell capture chamber, microfluidics for labelling/marker addition and cytometry channels and fluorescent counting.
- Selection and implementation of immunological techniques for identification of cellular species of interest, e.g. *E. coli*, with subsequent benchmarking against standard reference methods. A key feature of the proposed system includes labelling with immunological recognition elements to allow more specific online identification of target species than currently achieved by remote systems. The first target for detection was *E. coli* due to the availability of commercial immunoassay kits for system comparison and the availability of environmental samples containing detectable levels of the bacterium. It is envisioned that following thorough system evaluation, modification of the immunoassay protocol will allow the detection of

other targets of interest, including toxic algae and *Cryptosporidium*.

- Investigation of enhancing the period of use of the proposed system without manual intervention. Assessment of potential risks to system operation from clogging and biofouling includes the use of fresh and marine samples with varying degrees of particulate loading. The use of coatings and localised 'bubble-assisted' scrubbing to remove build-up of growth, along with an alternative clean-in-place (CIP) chemical treatment as part of the cleaning cycles, was a potential approach for consideration along with illumination of system with UV light.

A list of specific work packages (WP) and the associated deliverables (D) is outlined in Fig. 1.1.

The outputs of Tyndall-led WP1, WP2, WP3 and WP6 are outlined in Chapter 3 of this report. Chapter 4 details the work of partners in ZEPS on immunological characterisation and Chapter 5 details investigations related to marine biofouling. Appendix 1 details work packages and deliverables and Appendix 2 details additional outputs of the research in the form of presentations and publications.

As a result of operational difficulties encountered in the work programme (as outlined in Section 3.0 of the End-of-Project Report), a fully functioning prototype system has yet to be demonstrated, preventing the completion of the following deliverable reports:

- D2.3 Updated parts throughout project period
- D3.3 Final specifications and design files
- D4.2 Report on performance of unit under differing conditions and in light of system modifications and updates, and
- D5.1 Report on effectiveness of options and improvements obtained.

Recent progress was made in the heterogeneous integration of the detector array and in the integration

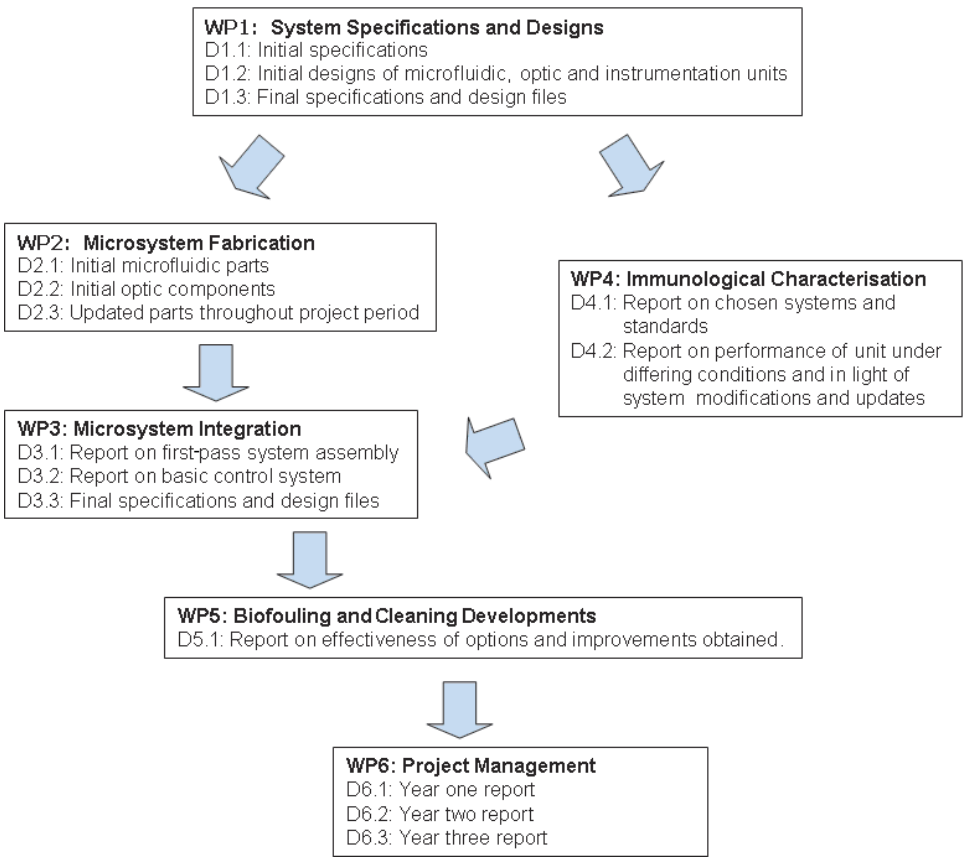



**Figure 1.1. Overview of individual work packages (WPs) and associated deliverables (D).**

of the light source towards the end of the project and in light of this some new approaches to system design are under investigation. Due to the ongoing interest in the development of this technology platform, follow-on funding is now actively being sought from additional sources.

# 2 Overview of Technical and Commercial Literature

## 2.1 Commercial Bench-Top Systems

There are a number of commercial bench-top flow cytometry units available. The majority of systems in use are large-scale bench-top systems. These units are used by the medical and pharmaceutical industries and cost between €40k and €250k. Information on cell size is achieved through the use of scatter measurements. Cell viability is generally determined prior to cell counting through assessment of cell membrane integrity or metabolic activity. Pre-incubation with die solution (also termed marker) can show membrane integrity, e.g. Trypan Blue stain will only permeate and stain damaged cell membranes, indicating cell death. Metabolic activity is assessed through incubation with a salt, which is cleaved by healthy cells to form a colour product. Cell-sorting options are also available on some models.

More recently, manufacturers, such as Guava Technologies (http://www.guavatechnologies.com), have introduced smaller bench-top units that don't require the use of sheath-flow liquids or complicated optical alignments. This is a significant development as it greatly reduces the cost of the unit where a litre of sheath liquid is needed for every millilitre of sample analysed. The Guava system eliminates the need for sheath flow by using a size restriction for entry of cells to the counting chamber. The manufacturer also provides markers for cell viability assessment. This unit is not suitable for field applications, however, and retails for approximately €40,000.

Biodetect's microcyte unit (http://www.biodetect.biz) is available as a bench-top portable system. This approach uses miniaturised optical components, including avalanche photodiodes (APDs). The sheath fluid is recirculated in the system, which demands the use of cleaning solutions and the replacement of filters and fluids periodically. The application areas include monitoring of phytoplankton and of fermentation. Of the systems reviewed, many depend on a correlation with background chlorophyll fluorescence as opposed to specific cell identification. The Biodetect system uses chlorophyll auto-fluorescence as an indication that species such as *Cryptosporidium* may be present.

## 2.2 Research-Level Systems

Some recent review articles highlight developments in the area of microfluidic systems for cellular analysis (Huh *et al*., 2005). Different mechanisms are used to route and sort cells, including pneumatic pressure, electroosmotic flow, dielectrophoresis and the use of on-chip pumping mechanisms. The majority of microfluidic cytometry devices have been developed for clinical applications. Detection modes used for cell counts include fluorescence, spectral impedance and imaging with CCD (charge-coupled device) cameras. There are only a few research-stage systems that have successfully integrated all components needed for the development of a micro total analysis system for flow cytometry.

The flow cytometry chip previously developed at the Tyndall National Institute (Kruger *et al*., 2002) achieved cell counts and cell sorting on a microfluidic device. The microchip was fabricated on a silicon wafer using SU8 photoresist polymer and the channels were 50 µm in depth. The microchip then had to be diced and sealed to a glass slide. The integration of a high-gain APD was investigated through the use of flip-chip bonding. Indium gallium nitride (InGaN) light-emitting diodes (LEDs) were used for excitation but were not integrated into the device. A correlation was observed between the cross-sectional area of the incoming and outgoing channels and the resulting diffusion between the sample and the sheath fluid. Leaky waveguides and diffraction gratings were used to improve detection sensitivities.

## 2.3 Autonomous Remote Systems

There are two main systems in this area. CytoBuoy (www.cytobuoy.com) has a range of bench-top and remote systems available for phytoplankton analysis. Its desktop flow cytometer, CytoSense, costs between €40,000 and €60,000, depending on the system features required including the number of detectors,

etc. Its submersible version, CytoSub, is €16,000 extra and the CytoBuoy version is €29,000 extra. The instrument specification includes the ability to measure suspended microscopic particles, including phytoplankton and single cells. The maximum concentration is $10^{10}$ particles/l. Sampling throughput is listed as between 20 and 2 µl/s with a maximum sampling frequency of every 1.5 min, depending on particle loads. Maximum analysis rate is listed as 20,000 cells/s. A relatively high flow rate of 20 µl/s is used. For remote applications data transmission is achieved with line-of-sight radio transmission; however, satellite communications are under development. With the battery capacity available, the manufacturers state that 3,000 or more samplings can be carried out *per annum*, with an annual recommended system service to be carried.

The other main system of interest has been developed by Olsen *et al.* (2003) at Woods Hole Oceanographic Institute. The system is designed for the *in-situ* analysis of individual particles in sea water. The unit uses light scattering and fluorescence measurements for sample characterisation. A filtered sheath flow is also used in this design. The system has been designed to be moored, and the transmission of data from a remote location was not addressed in the citation referenced. Neither the CytoBuoy nor the Woods Hole system incorporates specific labelling of targets and both rely on a combination of scatter and background fluorescence measurement and correlation to determine species present.

# 3 Development of a Miniaturised Cytometry Platform

## 3.1 Introduction

The fabrication of the core system proposed the integration of optical components with a microfluidic channel network and was covered by WPs 1–3. A complete list of work package tasks and associated deliverables is outlined in Fig 1.1. Figure 3.1 details the main components of the proposed integrated system, including the fluidic channel, integrated optics, fluidic and optical control units, and the interface of all elements to a data output and processing system.

While progress towards the development of an alignment-free optofluidic system for cytometry was achieved, significant issues were encountered in relation to the packaging of the integrated unit which impacted on the resulting work programme. Heterogeneous integration of highly sensitive, space and power-efficient components (APDs and microLEDs) with microfluidic channels was investigated using specialist flip-chip alignment and attachment methods. While particle counts were not achieved in the final unit, a functioning detector array for integration with a fluidic channel was developed which would ultimately be useful for particle counting applications. Final outputs include new designs for the heterogeneous integration of key optical elements, which are currently being investigated for invention disclosure.

## 3.2 System Specifications and Design (WP1)

The system specifications detailed the initial designs of system components including microfluidic and optical elements. A significant literature review was prepared as part of the report. System specifications have been continually updated in light of technical progress to date. The preferred embodiment of the optical module design includes a light source and a detection element pre-aligned to a microfluidic channel.

## 3.3 Microsystem Fabrication (WP2)

This work package focused on the fabrication and processing of the components needed for system assembly and investigated the initial microfluidic elements and optic components selected for the proposed system.

### *3.3.1 Microfluidics*

A network of microfluidic channels ranging from 100 to 300 µm were fabricated in poly(dimethylsiloxane)

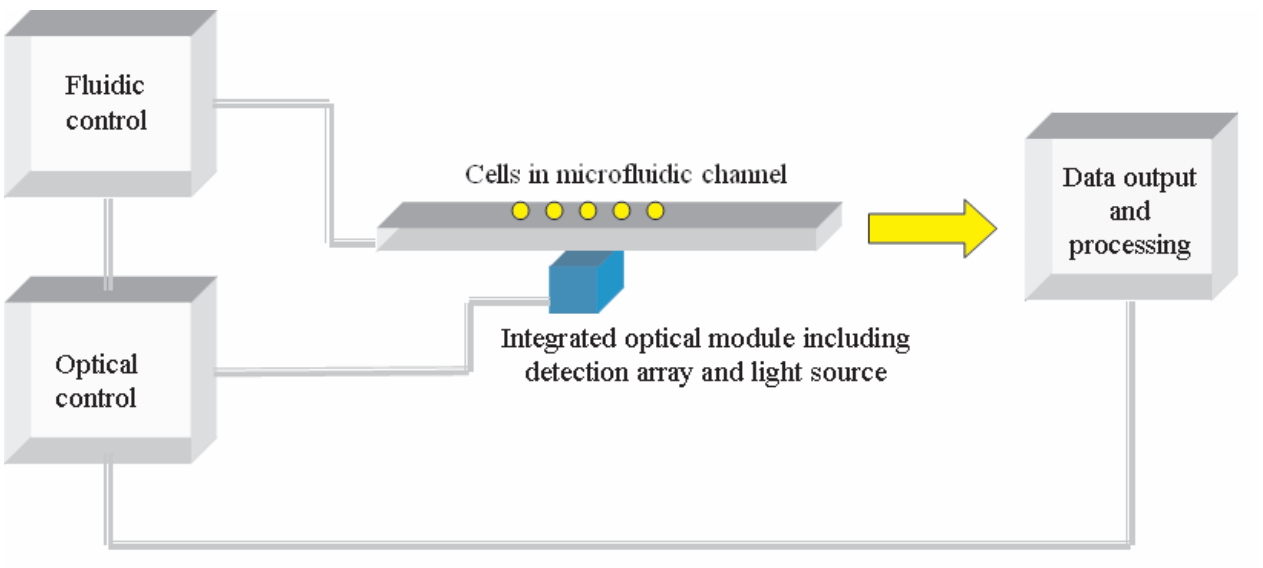



**Figure 3.1. Schematic of system layout.**

(PDMS). The use of PDMS substrate allows the production of inexpensive disposable polymer devices that offered significant advantages of low cost and easy modification during the initial design stage.

The process flow steps for fabrication of microfluidic devices are:

1. Design and print of layout mask
2. Fabrication of the replication template using photolithography
3. Casting of PDMS against the template and curing
4. Sealing of device layers using plasma treatment, and
5. Connection of fluidic inputs to syringe pumps.

For high-pressure applications, PDMS must be plasma treated in order to bond device layers and prevent leakage. Some issues were encountered with the reproducibility of sealing; however, these were resolved. The fluidic channels were controlled using two syringe pumps (one for sheath and one for sample delivery). The sheath flow line incorporated a tee to minimise the number of pumps needed. Ultimately, production of fluidic channels in SU8 or an alternative hard polymer is preferred and would also allow direct flip-chip attachment of optical components. This was not investigated during the development stage due to the higher cost of processing and the additional dicing steps that would be required.

The design incorporates a standard central channel through which the sample is introduced and side channels to introduce the sheath flow for sample focusing. The microchip has identical inputs and outputs, which could allow back-flushing of the chip if needed.

The successful focusing of a sample flow with sheath flows in the designed network is illustrated in Fig. 3.2. For visualisation purposes, fluorescein dye was added to the sample. A range of sample and sheath flow rates were investigated across the microlitres/second range. Some limitations on sample throughput were noted due to the nature of the PDMS material. Despite plasma treatment, device leakage was observed at elevated sample flows. The optimum ratio of sheath to sample flow was 10:1.

### *3.3.2 Detector array*

SensL included several cylindrical designs on its mask set and provided individual dies at no additional cost to the project. The initial designs were updated and included on a new mask set. Designs included passive and active quench capabilities.

The use of individual dies presented additional challenges in terms of the proposed laser processing. Issues with the laser drilling of optical components contributed to subsequent delays in WP3. Difficulties that resulted from issues involving the handling of single dies (overheating of individual samples and consequent damage during processing) were addressed in conjunction with the National Centre for

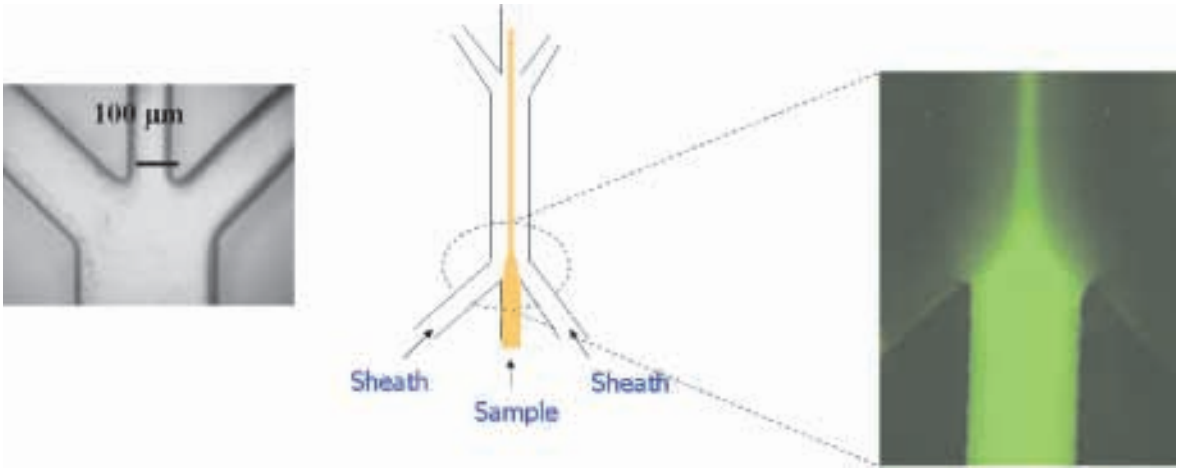



**Figure 3.2. Sample focusing in microfluidic channels.**

Laser Applications (NCLA), Galway, through the fabrication of holders designed to hold dies in place during processing to minimise damage. These issues would not be as significant if wafer-level processing were possible, which would be required for volume production. It is believed that these issues have since been overcome to some extent through optimisation of the die handling with development of polymer holders and the conditions of the laser drilling process as illustrated in Fig. 3.3.

While it is now apparent that the drilling of small batches of dies is highly problematic, it is anticipated that if performed on a mass fabrication scale this would be easier to implement. Probe station testing of fully packaged dies has demonstrated that laser-drilled dies are still operational following laser processing. Concerns with the relatively low dynamic range of the first batch of drilled dies were addressed by additional processing of dies from a new mask set.

### *3.3.3 Light source microLED*

The availability of suitable light sources for the project was explored. Sources available within Tyndall were initially limited to larger LEDs, which overshadowed the die array and were therefore likely to contribute to detector background. During the course of the project, researchers collaborated with the III-V materials and device group at Tyndall. This group has developed extensive technological expertise in light emitting and recently developed a microLED illumination source which became available for testing.

The microLEDs have greatly reduced emitting areas of approximately 15 µm. Maximum light extraction efficiency is achieved through a quasi-collimated emission beam to direct the light into the application zone. Increased switching speeds should provide reduced photobleaching of targets. Negligible heat generation allows the mounting of devices directly on microfluidic channels in the form of clusters and addressable arrays (Fig. 3.4). Attachment and

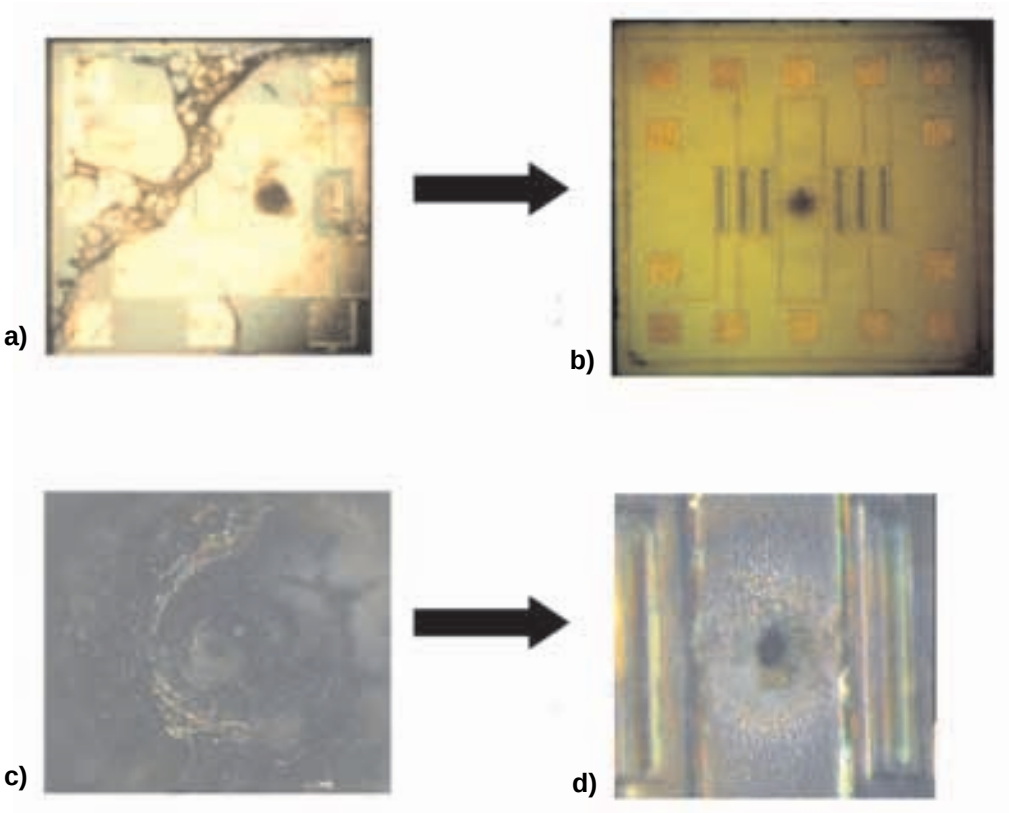



**Figure 3.3. Improvements in approaches to the laser drilling process. a) Image of drilled die with residue of Emulsitone™ coating. b) Image of die after optimisation of drilling procedure to remove need for coating. c) Image of pinhole and die fracture. d) Image of pinhole achieved using the optimised drilling procedure.**

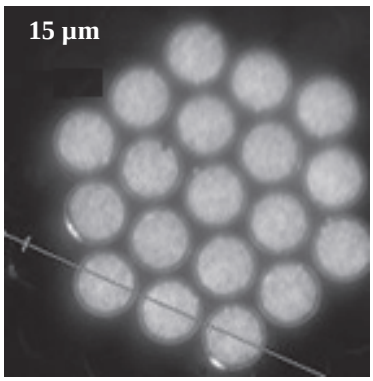



**Figure 3.4. Cluster of microLEDs.**

operation of microLEDs were investigated and will be discussed.

## 3.4 Microsystem Integration (WP3)

### *3.4.1 Packaging*

This work package focused on the integration of system components, described in the previous section, to enable cell analysis. The overall process flow for the production of the final system required multiple sequential processing steps for the production of a single system. The main issues encountered were due to detector processing. Packaging steps were carried out in Tyndall's specialist facility. Protocols were initially based on those used previously for other projects; however, significant adaptation and process optimisation were carried out for the cytometry application. The steps required for packaging of optical components are shown in Fig. 3.5. Optimisation of each packaging step was investigated. The final conditions used were detailed in relevant work package reports.

SensL designed a custom interface unit for operation of the APD array. Significant modification of the control boards and the interface board was carried out by SensL at no additional cost to the project. Figure 3.6 shows images of the gold tracking used to house the optical components and interface to the SensL control unit.

An active quench approach using resistors on the array was also investigated in order to achieve a higher dynamic range; however, further work is needed on this approach due to circuit parasitics. As a result a

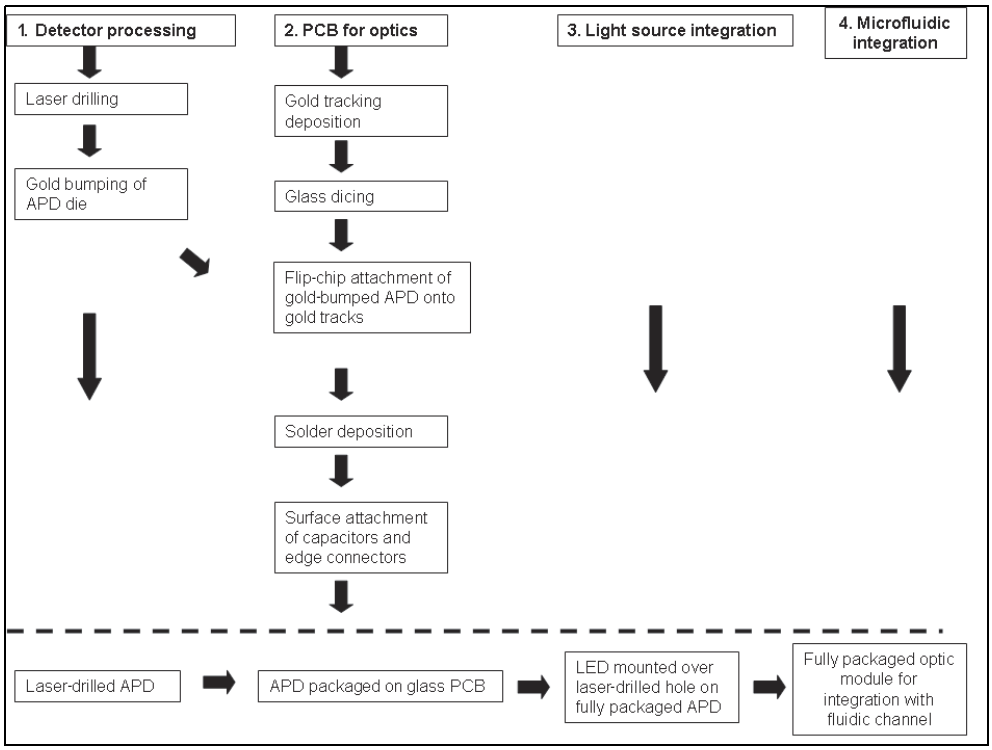



**Figure 3.5. Process flow for component integration into final system. (APD, avalanche photodiode; LED, light-emitting diode; PCB, printed circuit board.**

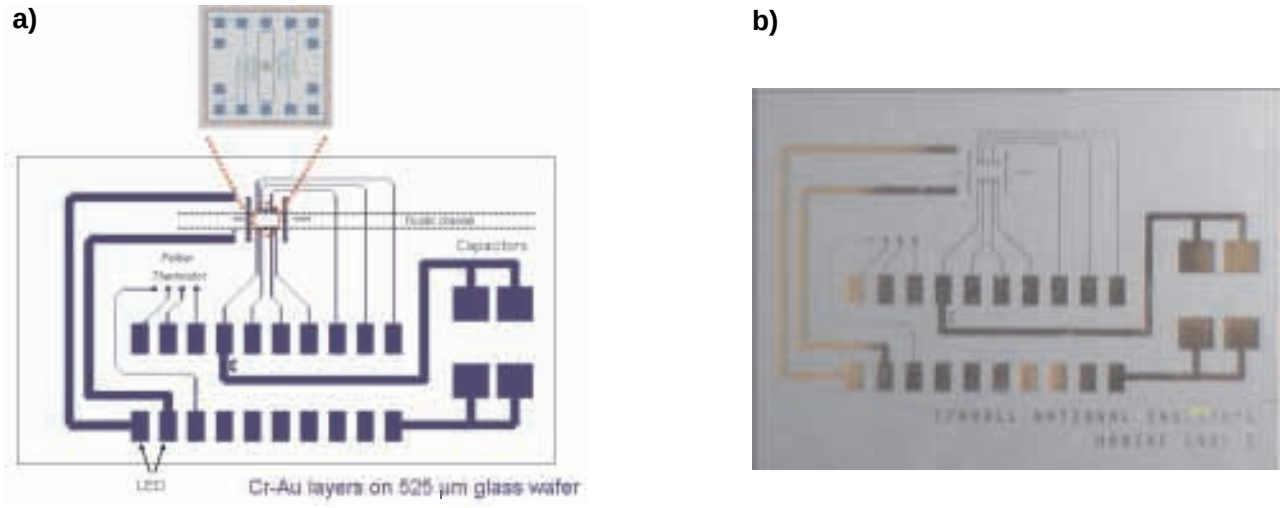



**Figure 3.6. a) System tracking and layouts for interface with the SensL unit; b) glass wafer with gold tracking.**

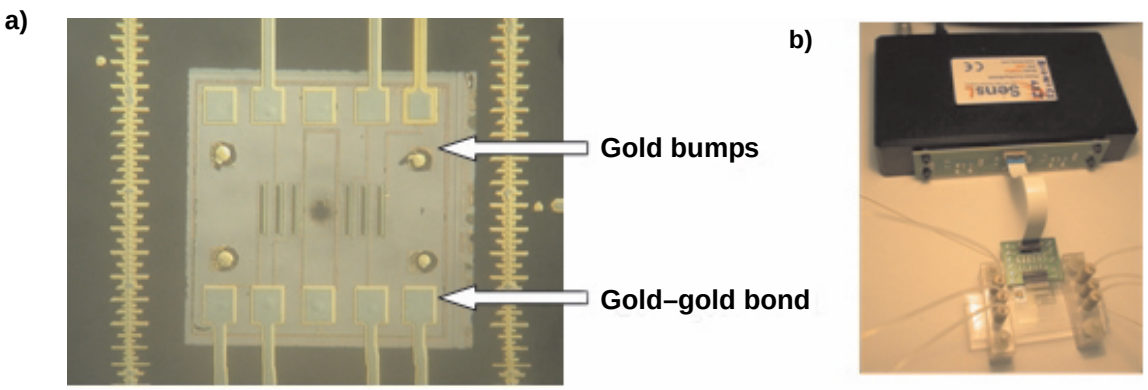



**Figure 3.7. a) Image of flip-chipped die array on transparent glass substrate. b) Custom-designed ribbon cable connection to the SensL unit.**

passive quench approach was investigated. Figure 3.7 illustrates a laser-processed detector and also the final device coupled to the SensL control interface using a ribbon cable approach.

A microLED was mounted on the back of an APD array to allow illumination of the channel. Control of the unit was achieved using an external power supply. Figure 3.8 shows the detection of light from the pulsed microLED, which was mounted on the back of the array.

Detection of light pulses was achieved with a second detector unit placed on the other side of the array to ascertain whether sufficient illumination of the microfluidic channel could be achieved. Figure 3.9 shows the output from a fully packaged array, with two of the active areas responding to a change in ambient light levels.

The reliability and lifetime of the gold–gold bonded die was a major issue, with packages only operational for a few days. This demonstrates that the proposed packaging did work; however, an improvement in the bonding procedure was needed.

As a direct result of reliability issues, an alternative solder attachment in addition to the gold bump was investigated. Diode testing (Fig. 3.10) illustrated that the die was operational and this would appear to be a viable alternative. In addition, back-up options looking at the use of optical fibres for light transmission were

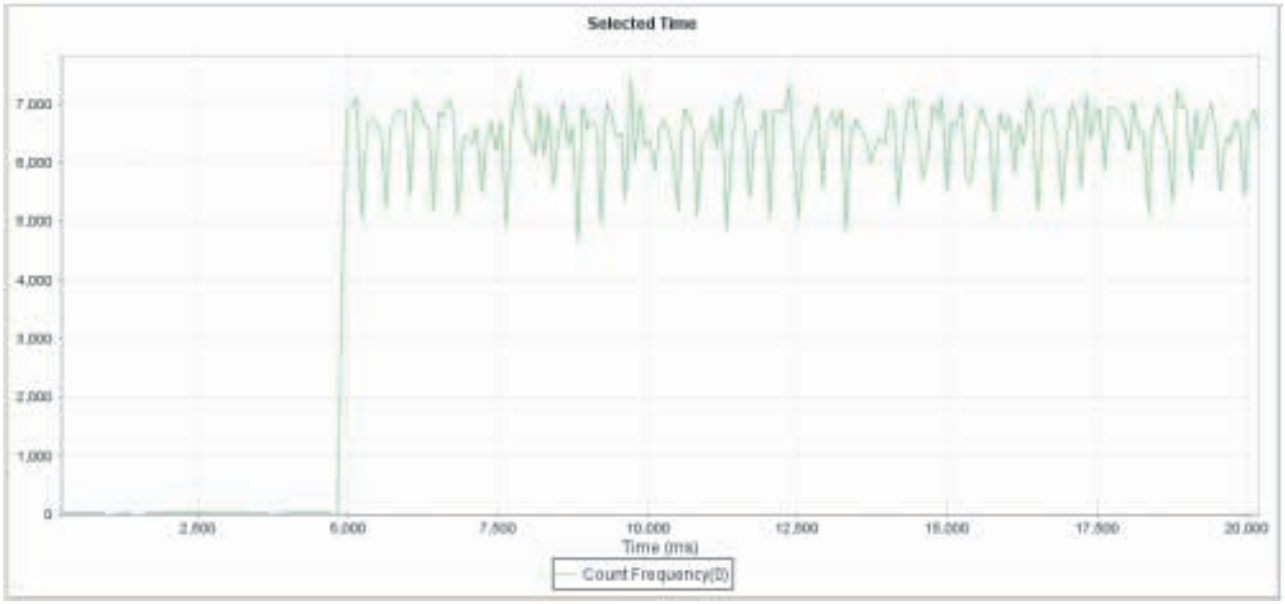



**Figure 3.8. Detection of microLED illumination through an avalanche photodiode array.**

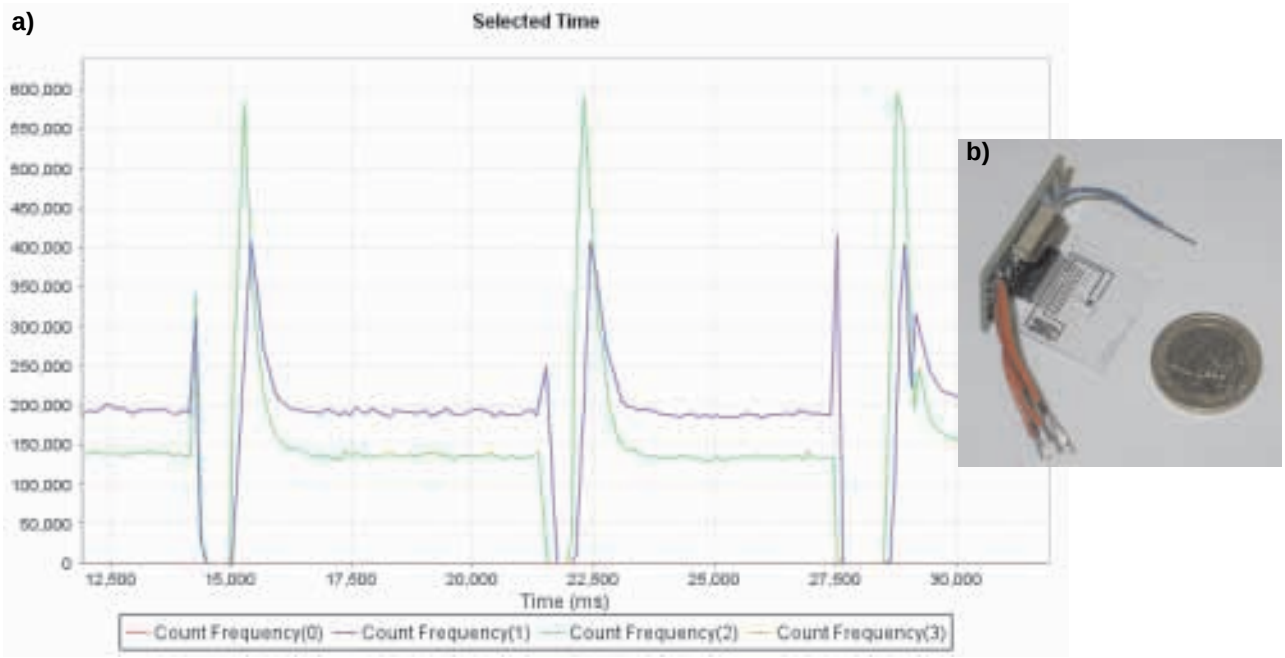



**Figure 3.9. a) Response from a fully packaged avalanche photodiode array; b) photo of an optical package.**

investigated, impedance electrodes were fabricated and preliminary testing was initiated.

Two back-up options were investigated due to the packaging difficulties encountered with the pinhole approach. The first investigated the use of fibre optics which had been investigated in the literature previously. A commercially available photon-counting module (PCM) mini was provided on loan from SensL. This unit has an APD aligned to the end of a fibre optic. The fibre optic was then inserted into a channel pre-aligned to the fluidic channel during the design phase and fabricated in a single run. The microLED was aligned with the end of the fibre optic and inserted into a corresponding channel aligned to the fluidic network

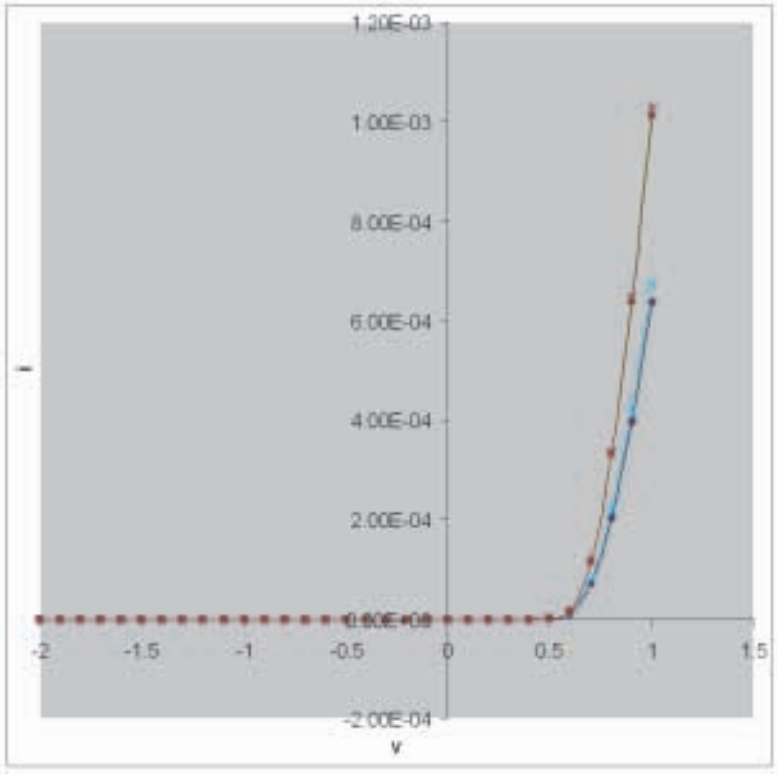



**Figure 3.10. Current–voltage (IV) curves for an avalanche photodiode array using a combination of gold bump and solder.**

(Fig. 3.11). Several alignments using different channels were investigated. While the unit did register a different count for fluorescent and non-fluorescent backgrounds, when fluorescent beads were introduced into the channel no significant change in response was recorded. Further optimisation of the set-up could include the addition of optical filtering in order to minimise background contributions from the microLED source.

An impedance option was also investigated as an alternative in conjunction with another project. Electrodes were deposited on glass and aligned with channels fabricated in polyimide during the fabrication procedure (Fig. 3.12). Device sealing was problematic and attempts to seal device layers resulted in clogging of fluidic channels with glue. Further work with this approach is needed but it does allow the potential of a label-free approach to bacterial detection.

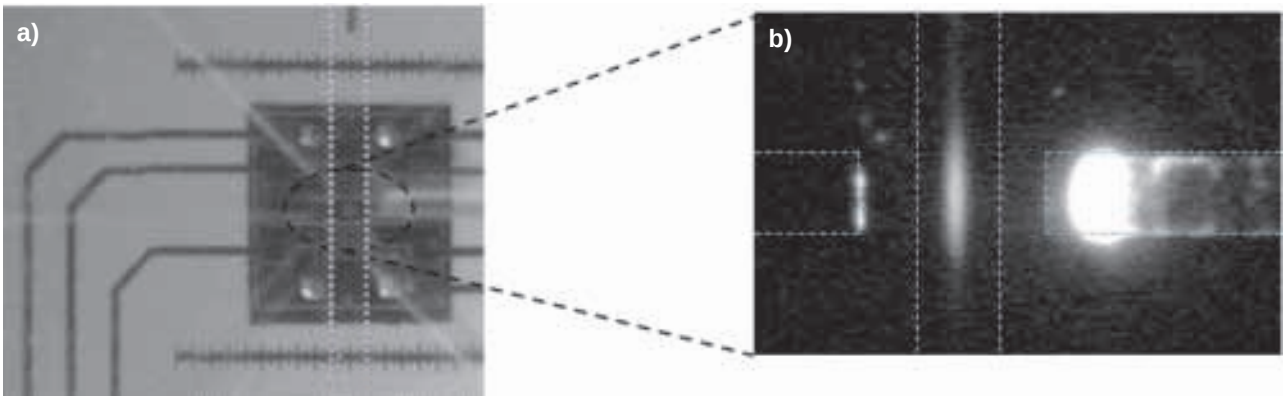



**Figure 3.11. a) Fibre optic inserted into poly(dimethylsiloxane) channel; b) illumination with light source.**

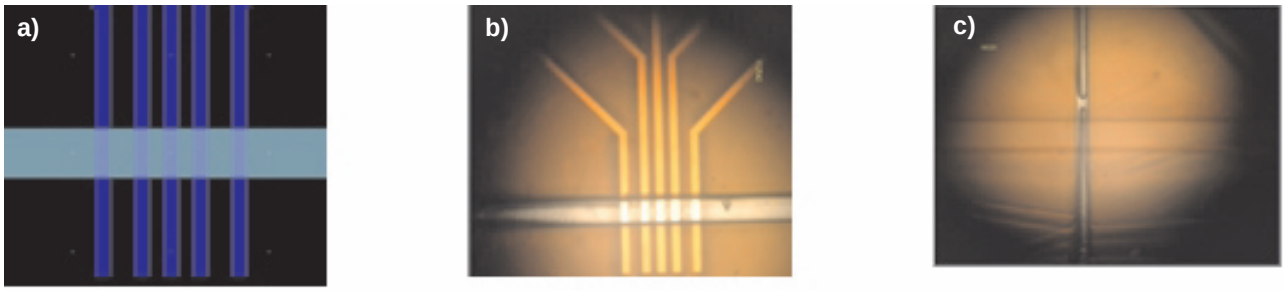



**Figure 3.12. a + b) Impedance-based detection chip; c) device-sealing issues.**

## 3.5 Integrated System Control

The development of a LabVIEW-based control sequence for operation of the micro-flow cytometry unit would allow integrated control of fluidic and optical components including LEDs, photodiode detectors, fluidic valves and syringe pumps interfaced to a microfluidic platform. The commercial software used controlled the fluidic system using syringe pumps (Kloehn, USA). The selected pumps have a 48,000-step resolution and integrated 8-way programmable valves. Syringes with volumes from 5 µl up to 50 ml can be used. A user-programmable memory allows control through an RS485 or RS232 serial interface. The units are modular, hence simultaneous operation of multiple devices can be achieved by storing commands for immediate or delayed execution.

Initial work has focused on the operation of the pumps using the supplied commercial software. Protocols were then transferred to the LabVIEW program for operation of the complete system. An easy user interface through a front-panel LabVIEW display was also developed (Fig. 3.13).

The software currently controls:

- Both the APD signal collection and the syringe pumps so that they work synchronously
- The simultaneous use of four channels of the APD

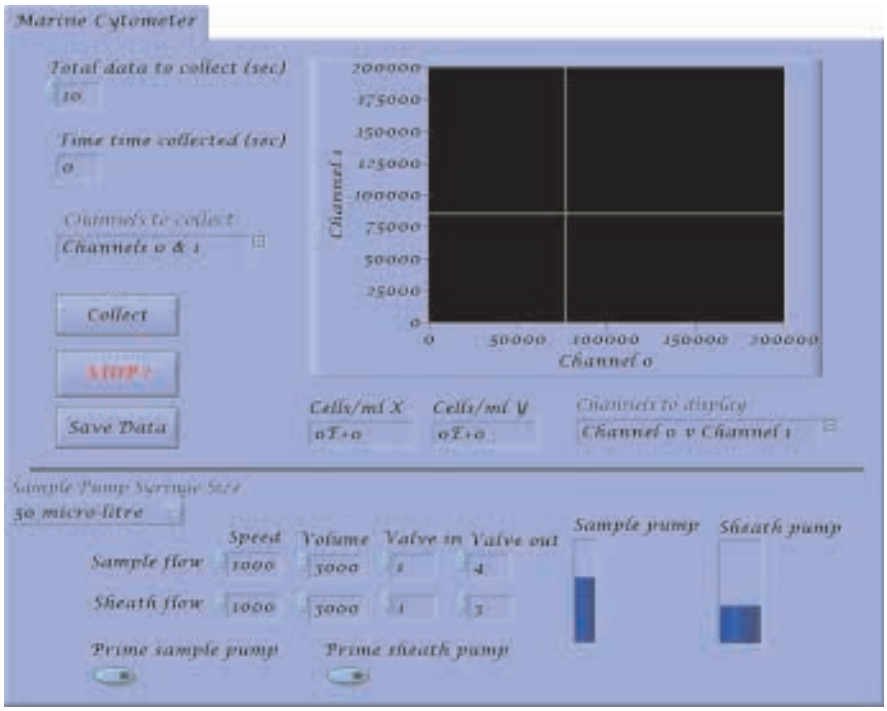



**Figure 3.13. LabVIEW-based control interface.**

- The use of a programmable threshold to define the mean photon count from the APD as a positive signal (i.e. a cell)
- The means whereby the data can be saved into a text file that can readily be opened in spreadsheet packages such as Excel, and
- The priming of syringe pumps.

Future versions of the software would include saved data to be re-displayed and manipulated, more rapid collection times for single channel collection and rapid throughput, and ultra-fast sampling using the APD in time-bin mode.

## 3.6 Key Conclusions

### *3.6.1 Microfluidics*

Poly(dimethylsiloxane) was initially explored as a substrate material for the fluidic channels due to its low cost and ease of use. This material is an optically transparent flexible polymer; however, in order to withstand the high back pressures generated during fluid pumping, plasma oxidation of the surfaces is required to achieve a reliable seal. Issues were encountered in connection with the reproducibility of plasma treatment, which led to some delay in the work plan. A new plasma oxidation chamber is currently on order and will be commissioned shortly. SU8 (photo definable spin-on polymer) on glass or alternative with flip-chipped optics is the preferred route to the manufacture of an alignment-free device.

### *3.6.2 Packaging*

An ultrasonic module for the FINETECH flip-chip instrument has recently been purchased. This unit is currently being commissioned and will add key capabilities in this area, including the option of thermo-sonic bonding. A new gold wire bonder allowing the fabrication of gold-coined wire studs has also recently been acquired. Initial experiments suggest that this will allow more reliable attachment of the optical components to PCB platforms, which is a key requirement of this kind of approach. In addition, key support for packaging capabilities at Tyndall have been realised due to the establishment of a new group focusing on the area of heterogeneous integration and optical packaging.

### *3.6.3 Final system*

A complete realisation of the system initially proposed has yet to be achieved. While the components are in place for assembly and first-pass testing of the optical unit, whether or not the proposed system will be sensitive enough to detect particles passing the detection zone has yet to be determined. Due to issues encountered with the initial pinhole approach, several other integrated module designs have been proposed as a direct output of this research. For proprietary reasons these new designs have not been included as part of this report. These approaches will be assessed for suitability to patent in the near future. The additional funding resources needed to complete system assembly and testing are currently being pursued through Enterprise Ireland Proof-of-Concept and other funding streams. Testing of system components is ongoing to determine if the output of the source is sufficient to illuminate the target (fluorescent beads). A wavelength-sensitive detector is being used to assess same. Optical filtering and output signal processing are also under investigation.

### *3.6.4 Key actions*

Several actions are needed to establish whether or not the proposed system will be sensitive enough to detect particles passing through the detection zone.

These include:

- Wavelength-specific testing of illumination output of fully packaged microLEDs as a function of operating power
- Testing of dark counts and noise background of fully packaged APDs
- Wavelength-specific detection of scatter and fluorescence generated by movement of microparticles and cells through the microfluidic interrogation zone
- Testing of a fully integrated system in light of improved packaging procedures
- Detection of particle scatter/fluorescence using a commercial APD system for reference and validation.

# 4 Immunological Labelling

This section of the project relates to WP4 and focused on the optimisation of immunological techniques for the detection of *E. coli* in suspension and their application in the development of a miniaturised flow cytometry system.

## 4.1 Introduction

The members of the family Enterobacteriaceae are defined as gram negative, aerobic or facultative anaerobic, rod shaped and non-endospore forming and include the species *E. coli* and *Enterobacter* (LaGier *et al*., 2005). *E. coli* is a normal colonist, and is the predominant facultative organism of the human gastrointestinal tract but it makes up a very small proportion of the total bacterial content (Todar, 2002), it is about 2 µm in length and 0.8 µm in width. From early in the 19th century, coliform bacteria, and more specifically *E. coli*, have been used as indicators of water pollution (US EPA, 2005). Coliform bacteria are found in diverse environments, but drinking water is not a natural environment for them so their presence indicates a threat to the sanitation of the water (Rompré *et al*., 2002). Current methods of detection for *E. coli,* for example, involve the use of microbiological or immunological-based techniques. These techniques are usually laboratory based, time consuming and there are limits to their sensitivity, and they would require modification to allow their application in a remotely deployed system.

The majority of microbiological procedures involve a concentration or enrichment procedure, which requires elevated temperatures and an incubation period, detection of the micro-organisms and often quantification. Immunological methods provide very specific labelling for detection, and antibody-based techniques allow high specificity through molecular-level biological recognition, even if there is a high level of contamination, such as debris, present in the sample. These antibody methods provide a means of identifying micro-organisms without the requirement of culturing (Veal *et al*., 2002). However, many immunological methods require pre-culturing of the bacteria of interest prior to staining to ensure the presence of adequate numbers in the sample. Immunological-based methods have been modified and applied to a range of techniques such as fluorescent microscopy and fluorescent *in-situ* hybridisation, and have applications in other systems such as flow cytometry. Flow cytometry has the ability to measure multiple parameters on individual cells at environmentally representative numbers (Porter *et al*., 1996). The flow cytometer quantitatively measures the optical characteristics of cells as they pass in single file in front of a focused light beam (Veal *et al*., 2002). Light scattering as the laser bounces off the cells gives details regarding the cell's physical characteristics. An objective of the present study was to develop a flow-cytometry-based detection method for the screening of *E. coli* that could ultimately be transferred to a remotely deployed system.

## 4.2 Development and Maintenance of Bacterial Cultures and Evaluation of Current Microbiological Field Techniques

### *4.2.1 Cell culturing and preservation*

*E. coli* K12, an attenuated laboratory strain and *E. coli* WT555, an environmental isolate, were obtained from the Microbiology Department, UCC, and were used as test organisms. The *E. coli* were inoculated into Tryptic Soy Broth (TSB, Sigma–Aldrich) and incubated overnight in a water bath at 37°C. Growth cultures of the bacteria at concentrations of approximately $8 \times 10^8$ cells/ml were maintained in broth at 4°C for up to 2 weeks before being discarded.

### *4.2.2 Spectrophotometry/Turbidity method for the indirect enumeration of suspended bacteria*

This procedure was conducted using the spread-plate microbiological method and spectrophotometry to construct a standard growth curve of a bacterial species.

Serial dilutions were performed on the growth culture every hour for 10–12 h and the microbiological and

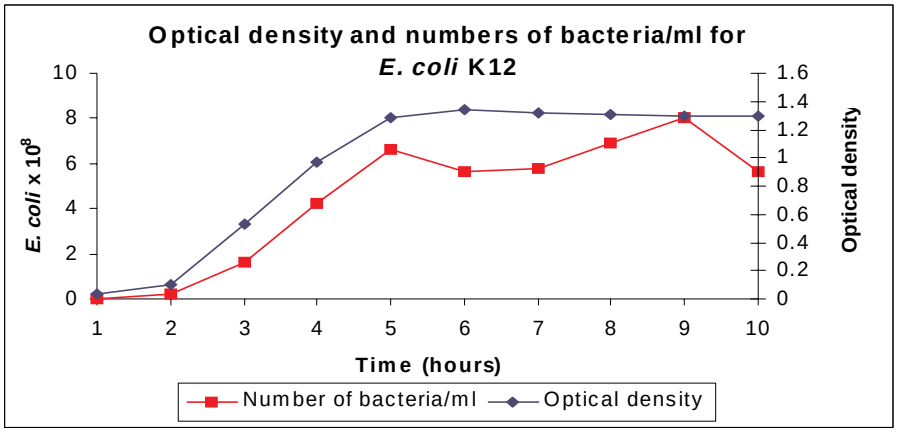



**Figure 4.1. An example of a typical standard curve generated showing the optical density of bacterial culture *Escherichia coli* K12, relative to the concentration of bacteria, determined through indirect culture methods.**

spectrophotometric results allowed the relationship between bacterial colony number and absorbance to be graphed.

#### *4.2.3 Results*

The bacteria were successfully preserved uncontaminated for the 2-year duration of the study. Cultures of grown *E. coli* were maintained in broth at 4°C for experimentation and were discarded after a 2-week period.

The standard curves (Fig. 4.1) were constructed to allow enumeration of subsequent cultures of *E. coli* K12 and WT555 by measuring the optical density of the log phase bacterial culture and comparing it to the graph showing the number of bacteria/millilitre for each hour counted and calculated using the spread-plate method.

## 4.3 Optimisation of Two Immuno-fluorescent Antibodies for the Detection of *Escherichia coli* Using Immunofluorescence Microscopy and Flow Cytometry

#### *4.3.1 Antibodies and reagents*

Two anti-*E. coli* fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) polyclonal antibodies (Europa Bioproducts (catalogue no. FZ60-E13) and AbD Serotec (catalogue no. 4329-4916)) were tested using immunofluorescence (IF) microscopy and flow cytometry. A rabbit-IgG-FITC isotype control was purchased from Abcam (ab37406). A number of *E. coli* bacterial strains were stained using fluorescent antibodies: *E. coli* K12, an attenuated lab strain; *E. coli* wild type 555, an environmental isolate; *E. coli* MKH13, wild type, a derivative of K12 but genetically dissimilar; and *E. coli* NCIMB-1943, an American Type Culture Collection (ATCC) strain. Staining was carried out on acetone-fixed cells on slides and in suspension (Fig. 4.2).

## 4.4 Optimisation of Staining Protocol for Unfixed Cells in Suspension Using Immunofluorescence Microscopy and Flow Cytometry

#### *4.4.1 Methods*

- Immunofluorescence microscopy was carried out using an Olympus camera and fluorescent microscope model BX51 TRF at 488 nm (blue filter) at ×200 and ×400 magnification. Photographs of the cells at different fields of vision were taken in phase and, using the fluorescent filter, these were examined later and an estimation of the percentage staining was obtained.

- A Becton Dickinson FACSCanto was used to perform flow cytometry on heat-fixed propidium-iodide-stained cells.

#### *4.4.2 Statistical analysis*

Statistical analyses were performed using the Brodgar software for univariate and multivariate analysis and

multivariate time series analysis, version 2.5.3. Data exploration and univariate statistics were executed on the data; linear regression analysis, *t*-tests and analyses of variance (ANOVAs) were also used on the data. Correlations were performed using Pearson's correlation on SPSS, version 12.0.1 for Windows.

### *4.4.3 Results*

#### *4.4.3.1 Immunofluorescence microscopy*

Overall, there was no significant difference between the numbers of *E. coli* K12 and *E. coli* WT555 stained (ANOVA: F = 0.9947, df = 1, $p = 0.3215$).

The highest percentage staining was obtained at a concentration of 1:10 of antibody (Fig. 4.3). The use of blocking agents in conjunction with phosphate-buffered saline (PBS) for washing and incubation of cells gave a significantly higher percentage staining of *E. coli* K12 (*t*-test: $t = -1.9$, $p < 0.001$) and *E. coli* WT555 (*t*-test: $t = -2.8$, $p < 0.001$) than antibody

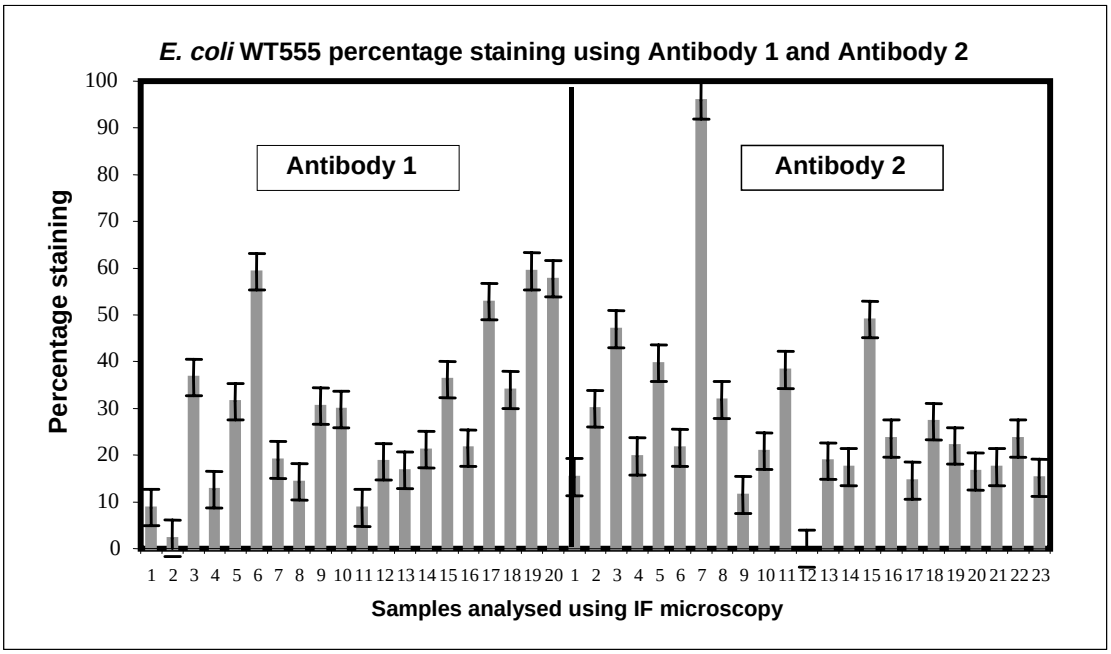



**Figure 4.2. The percentage of *Escherichia coli* WT555 stained using the two FITC-conjugated anti-*E. coli* antibodies. (IF, Immunofluorescence microscopy.)**

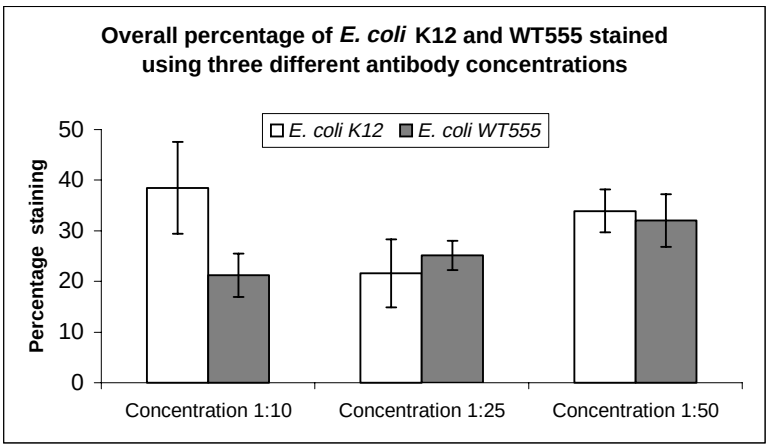



**Figure 4.3. The percentage of *Escherichia coli* K12 and *E. coli* WT555 staining at three different antibody concentrations, 1:10, 1:25 and 1:50, and examined using immunofluorescence microscopy.**

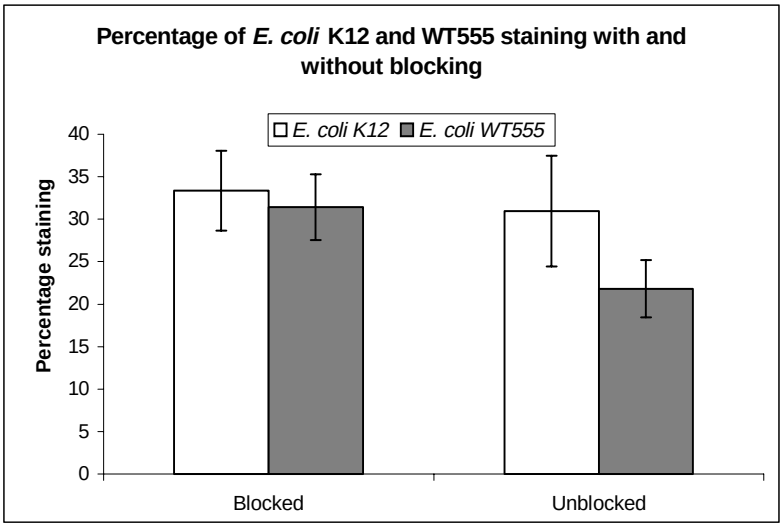



**Figure 4.4. The effect of blocking using bovine serum albumin and rabbit serum on the overall percentage staining of *Escherichia coli* K12 and *E. coli* WT555. The bacteria were enumerated using immunofluorescence microscopy.**

staining without using bovine serum albumin (BSA) and rabbit serum (RS) (Fig. 4.4).

#### *4.4.3.2 Flow cytometry*

Statistically, there was no significant difference in overall staining using the two commercial antibodies: 5.8% of *E. coli* K12 to 14.9% of the WT555 population (*t*-test: $t = 2.19$, $p < 0.05$) (Fig. 4.5).

#### *4.4.3.3 Washing and blocking steps*

There was a significant difference between the PBS and PBS/BSA washing steps (*t*-test: $t = –4.5$, $p < 0.0001$) but there was no difference between washing method 3 (PBS/BSA/RS) and method 1 (PBS) (t-test: $t = –1.182$, $p = 0.243$) (Fig. 4.6).

#### *4.4.3.4 Specificity of antibodies*

Stained bacteria were examined using both IF microscopy and flow cytometry. No binding was evident upon examination of *Enterobacter aerogenes* using IF microscopy and flow cytometry. There was no evidence of non-specific binding using *Bacillus subtilis*.

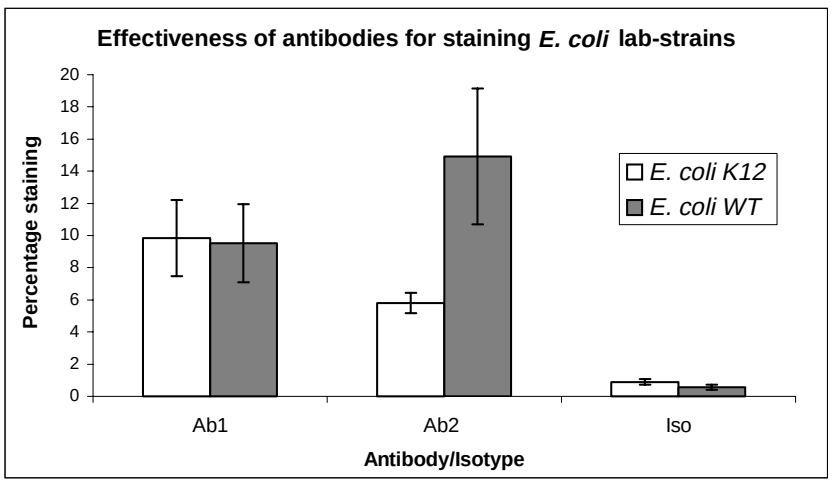



**Figure 4.5. The percentage staining of *Escherichia coli* K12 and WT555, using two commercial antibodies, and the isotype control detected using flow cytometry. (Ab1, Antibody 1; Ab2, Antibody 2; Iso, isotype control.)**

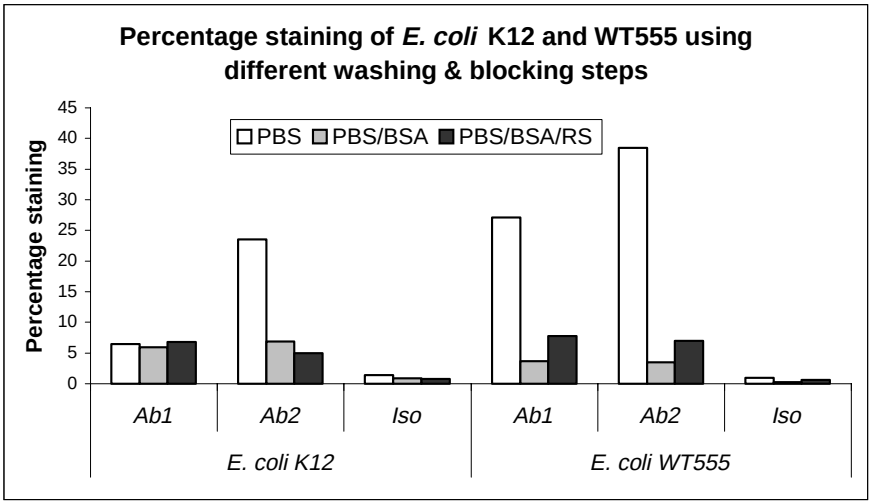



**Figure 4.6. Average percentage staining of *Escherichia coli* K12 and WT555 detected by flow cytometry using the different washing/blocking combinations on each of the antibodies. (BSA, bovine serum albumin; PBS, phosphate-buffered saline; RS, rabbit serum; Ab1, Antibody 1; Ab2, Antibody 2; Iso, isotype control.)**

Both IF microscopy and flow cytometry showed weak antibody affinity for *Bacillus cereus.*

#### *4.4.3.5 Mixed bacterial cultures*

An increase in percentage bacteria staining occurred as the concentration of *E. coli* in each sample also increased. Significant correlations occurred between the different bacterial mixtures, using Pearson's correlation (Table 4.1). The mixtures of *E. coli* K12 with *E. aerogenes* and with *B. subtilis* produced a highly significant correlation ($r = 0.863$, $p < 0.01$). There was also significant correlation between the staining of *E. coli* K12 mixed with *B. subtilis* and *E. coli* WT555 mixed with *E. aerogenes* ($r = 0.647$, $p < 0.01$). This demonstrates that despite the low staining achieved by the antibodies in pure *E. coli* cultures, a proportional quantity of bacteria were being stained.

*E. coli* MKH13 and *E. coli* NCIMB-1943 were both stained using the two commercial antibodies and examined *via* flow cytometry. Both bacteria were found to give a high percentage staining: 43% of *E. coli* NCIMB-1943 and 62.7% NCIMB-1943.

**Table 4.1. Result of correlations carried out between each of the bacterial mixtures stained: correlation between equivalent concentrations of *Escherichia coli* K12 and E. coli WT555 in mixtures of non-target bacteria.**

| Bacterial mixtures | K12 and *Enterobacter aerogenes* | K12 and *Bacillus subtilis* | WT and *Enterobacter aerogenes* | WT and *Bacillus subtilis* |
|---|---|---|---|---|
| **K12 and *Enterobacter aerogenes*** | | $r = 0.863$<br>$p < 0.01$ | $r = 0.638$<br>$p < 0.05$ | $r = 0.506$<br>Not significant |
| **K12 and *Bacillus subtilis*** | $r = 0.863$<br>$p < 0.01$ | | $r = 0.647$<br>$p < 0.01$ | $r = 0.527$<br>$p < 0.05$ |
| **WT and *Enterobacter aerogenes*** | $r = 0.638$<br>$p < 0.05$ | $r = 0.647$<br>$p < 0.01$ | | $r = 0.347$<br>Not significant |
| **WT and *Bacillus subtilis*** | $r = 0.506$<br>Not significant | $r = 0.527$<br>$p < 0.05$ | $r = 0.347$<br>Not significant | |

## 4.5 Detection of Water-Borne *Escherichia Coli* with Flow Cytometry and Immunofluorescence Microscopy Using Two Antibodies, and Comparison with Membrane Filtration

A wild type strain of *E. coli* 555 was isolated from an environmental sample by the Department of Microbiology, UCC, and was used as the control test organism. The bacteria were maintained in TSB as previously described and the two antibodies as previously described were used in flow cytometry and in IF microscopy.

Three different locations at the Cork City Lough (The Lough) and two locations on the River Lee, at the Angler's Rest and the Carrigrohane Road, were sampled every week over a 4-week period and four sites were monitored at each location, apart from Week 1 at the Carrigrohane Road, when only three sites were sampled (Figs 4.7–4.9). Water sampling was carried out between 8.30 and 9.30 am during August 2007. A sterile sample cup and a 2-m sample cable were utilised to take water samples at the sites. The water was then decanted into sterile 250-ml sampling bottles. All samples were transported to the laboratory, stored at 4°C, and processed within 8 h.

### *4.5.1 Heterotrophic plate count*

This procedure was done in order to ascertain the total number of heterotrophic bacteria present in the water samples, employing the protocol from *Standard*

**Figure 4.7. Cork City Lough.**

**Figure 4.8. The Angler's Rest.**

**Figure 4.9. The Carrigrohane Road.**

*Methods for the Examination of Water and Wastewater* (Clesceri *et al.*, 1998).

### *4.5.2 Membrane filtration*

For membrane filtration, membrane lauryl sulphate broth (MLSB, Oxoid) was used for the growth of *E. coli.* To confirm the presence of *E. coli* bacteria on membranes, two yellow colonies from separate replicates for each site were sub-cultured to a test tube of tryptone water.

### *4.5.3 Antibody labelling of cells*

Antibody labelling of cells using the Serotec antibody Antibody 1 (Ab1), the Europa antibody Antibody 2 (Ab2) and the isotype control was carried out. The antibodies and isotype were made up to a concentration of 1:25 in BSA (1%)/RS (20%)/PBS and 10 µl of the dilution were added to each of the round-bottomed tubes. The tubes were vortexed briefly to mix the antibody and incubated in the dark at room temperature for 30 min. A 3-ml volume of BSA (1%)/PBS was added to each round-bottomed tube and was centrifuged to wash cells, and this procedure was repeated. The cells were re-suspended in 50 µl of BSA (1%)/PBS and placed on ice until flow cytometry and IF microscopy.

### *4.5.4 Methods*

- Cells from 10 µl of stained cell solution were examined using an epifluorescent phase microscope at 488 nm (blue filter) at ×400 magnification.
- Flow cytometry was carried out on a Becton Dickinson FACSCanto, a six-colour bench-top analyser capable of analysing 10,000 events per second.
- In the final week of sampling, a further sub-culturing of *E. coli* species, that had been confirmed using microbiological methods, was performed from each location and stained using Ab1, Ab2 and the isotype control as per the above protocol. The cells were examined using flow cytometry.

### *4.5.5 Results*

#### *4.5.5.1 Heterotrophic plate count*

Figure 4.10 depicts the numbers of heterotrophic bacteria detected over the weeks and locations sampled. Levels of bacteria varied from week to week. Week 3 at all three locations produced the highest numbers of bacteria present per millilitre, in particular at The Lough, with the lowest levels at the

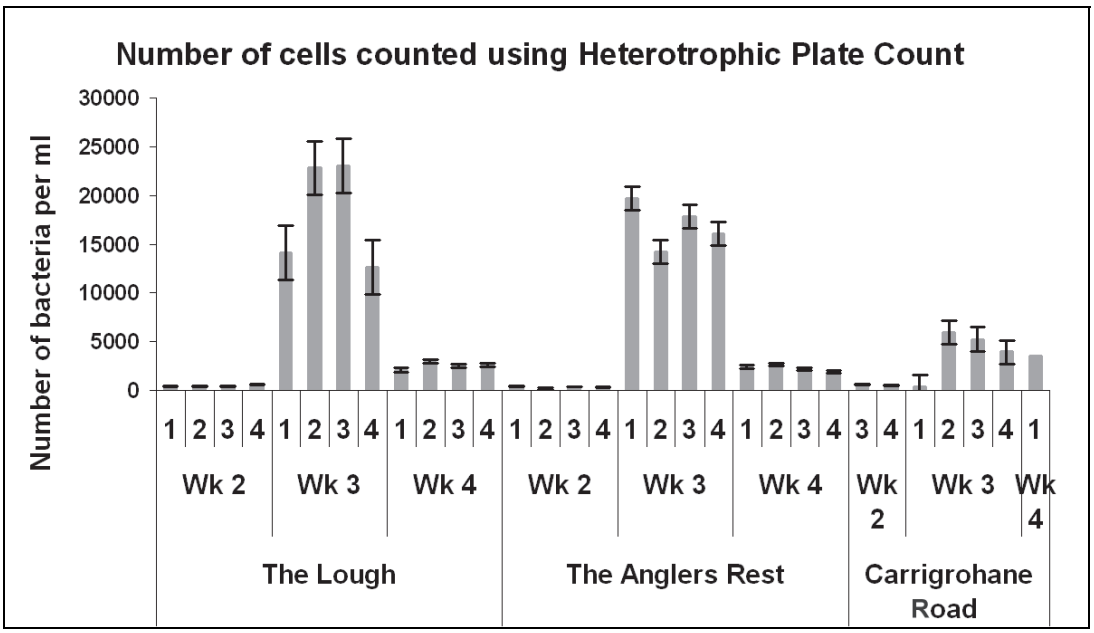



**Figure 4.10. The number of heterotrophic cells (mean ± standard error) counted from each uncontaminated filtered and incubated sample, 31 in total.**

Carrigrohane Road during this period. There was a significant difference in bacterial numbers between locations sampled ($p < 0.0001$, ANOVA) and between sites sampled within locations ($p > 0.01$). The average number of cells counted using the heterotrophic plate count method was 5,700 cells/ml.

#### *4.5.5.2 Membrane filtration*

Significant differences were present in bacterial numbers between locations sampled ($p < 0.001$), with The Lough having the highest percentage of bacteria confirmed as *E. coli* and the Angler's Rest having the lowest percentage of *E. coli*.

#### *4.5.5.3 Immunofluorescence microscopy*

The percentage of the total population staining was calculated and the average staining of Ab1 and Ab2 was 6.3% of the total bacterial population. There were no significant differences between sites or weeks examined using IF microscopy. There was a significant difference between sampling locations ($p > 0.01$, ANOVA) and between antibodies ($p < 0.0001$, ANOVA).

Table 4.2 illustrates the results of statistical analysis using ANOVA on the detection methods used for investigation of bacterial numbers. The difference between sites, locations and weeks was examined for each method and the difference between antibodies was examined for IF microscopy and flow cytometry.

#### *4.5.5.4 Flow cytometry*

Enumeration of stained cells using flow cytometry displayed The Lough with the highest percentage of *E.*

**Table 4.2. Comparison of counts between sites using different methods. Significance codes: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, not significant.**

| Method | Factors | df | F value | *p* (>F) |
|---|---|---|---|---|
| **Heterotrophic plate count** | Site number | 3 | 3.1548 | 0.03577* |
| | Week number | 3 | 0.0926 | 0.96365 ns |
| | Sampling area | 2 | 35.9379 | 1.733e-09*** |
| **Membrane filtration** | Site number | 3 | 2.2899 | 0.09386 ns |
| | Week number | 3 | 0.557 | 0.64663 ns |
| | Sampling area | 2 | 20.3357 | 9.894e-07*** |
| **Immunofluorescence microscopy** | Site number | 3 | 0.1473 | 0.93117 ns |
| | Week number | 3 | 0.6962 | 0.50098 ns |
| | Sampling area | 2 | 4.2192 | 0.01756* |
| | Antibody type | 2 | 38.8979 | 4.561e-13*** |
| **Flow cytometry: stained bacteria** | Site number | 3 | 4.1043 | 0.008067** |
| | Week number | 3 | 30.4084 | 5.831e-15*** |
| | Sampling area | 2 | 56.9696 | <2.2e-16*** |
| | Antibody type | 2 | 50.3727 | <2.2e-16*** |
| **Flow cytometry: total bacteria** | Site number | 3 | 2.7195 | 0.0472067* |
| | Week number | 3 | 31.8614 | 1.662e-15*** |
| | Sampling area | 2 | 9.3477 | 0.0001611*** |
| | Antibody type | 2 | 0.2011 | 0.818083 ns |

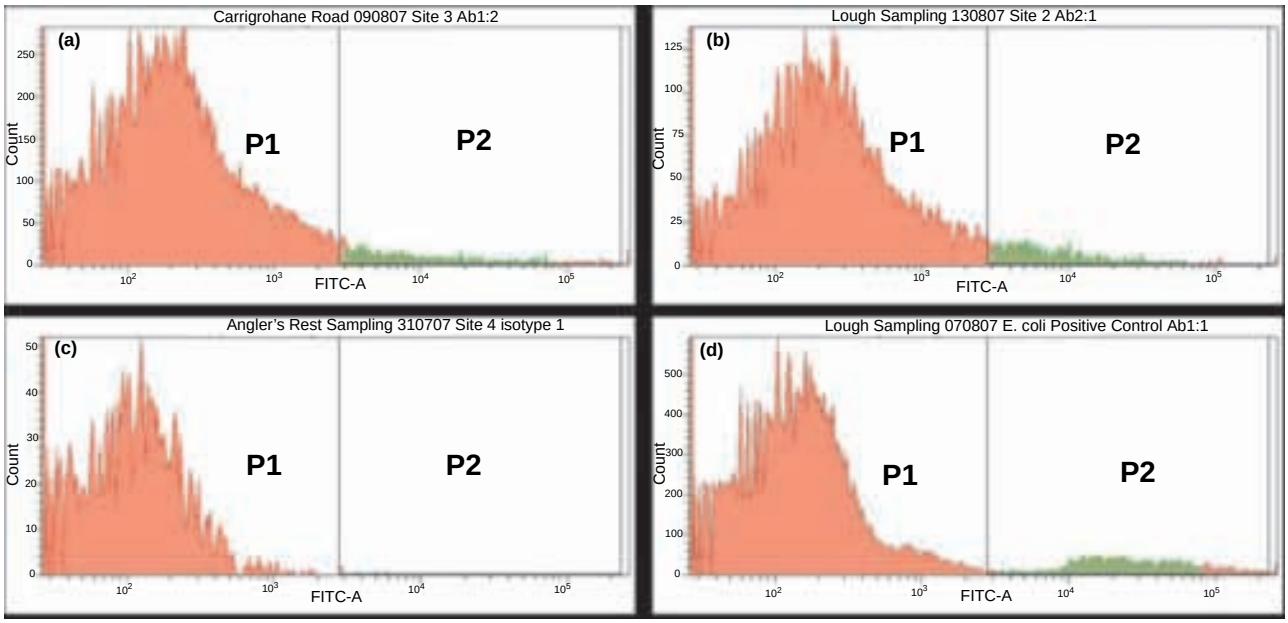



**Figure 4.11. Graphical output from flow cytometry showing the staining of bacteria at the different locations sampled using (a) Antibody 1, Carrigrohane Road, (b) Antibody 2, The Lough, (c) isotype control, the Angler's Rest, and (d) *E. coli* WT555, The Lough, as control test organism.**

*coli*, followed by the Carrigrohane Road, and the least amount of *E. coli* was detected at the Angler's Rest (Table 4.2). There was a significant difference between sites, weeks and locations using ANOVA (see Table 4.2). Further analysis using linear regression showed a significant difference between Site 3 and the other sites sampled at each location (*t*-test: $t = 0.06431$, $p < 0.05$). Flow cytometry allowed the stained *E. coli* to be distinguished from the other similarly shaped bacteria in the samples (Fig. 4.11).

### *4.5.6 Comparison of immunological and microbiological methods*

Figures 4.12–4.14 illustrate the percentage of *E. coli* detected using the three enumeration methods over the sites and weeks sampled. A comparison of the two antibodies showed no significant difference between their staining efficiencies.

The Lough had the highest proportion of *E. coli* present and the Angler's Rest had the lowest level of *E. coli* detected for each method apart from IF microscopy staining with Ab2, which determined that the Carrigrohane Road had the lowest proportion of *E. coli* present (5.2%) and The Lough the highest (6.6%). Membrane filtration detected the lowest proportion of *E. coli* and IF microscopy the highest. Correlations were carried out between the three methods using Pearson's correlation. Membrane filtration was positively correlated to flow cytometry ($r = 0.389$, $p < 0.01$).

Flow cytometry was positively correlated to IF microscopy ($r = 3.12$, $p < 0.01$). There was no significant correlation between membrane filtration and IF microscopy using Pearson's correlation.

## 4.6 Optimisation of an Automated Washing and Antibody Staining Filtration System for the Detection of Specific Bacteria *in situ* in the Environment

### *4.6.1 Bacteria, antibodies and reagents*

All bacteria, antibodies and reagents employed were as previously described.

#### *4.6.1.1 Kloehn filter pump*

The Kloehn model 50120 Intellect valve is a programmable fluid control instrument; the system has a non-volatile user program memory, which can retain stored information when not powered, and system expansion input/output. Included in the unit is a valve mechanism driven by a motor and software to allow

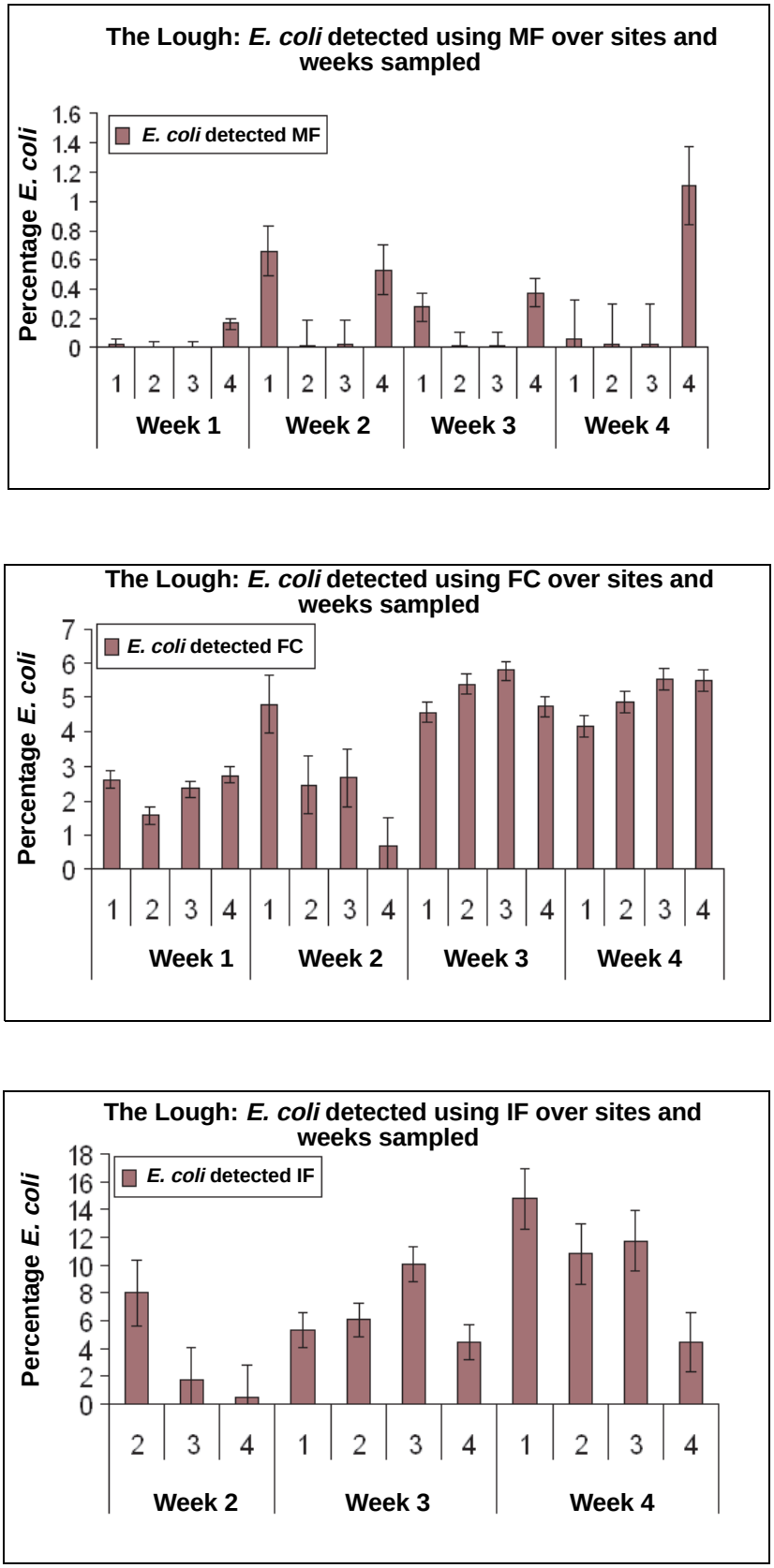



**Figure 4.12. The percentage of *Escherichia coli* detected at The Lough using each of the three detection methods over the sites and weeks sampled. (FC, flow cytometry; IF, immunofluorescence microscopy; MF, membrane filtration.)**

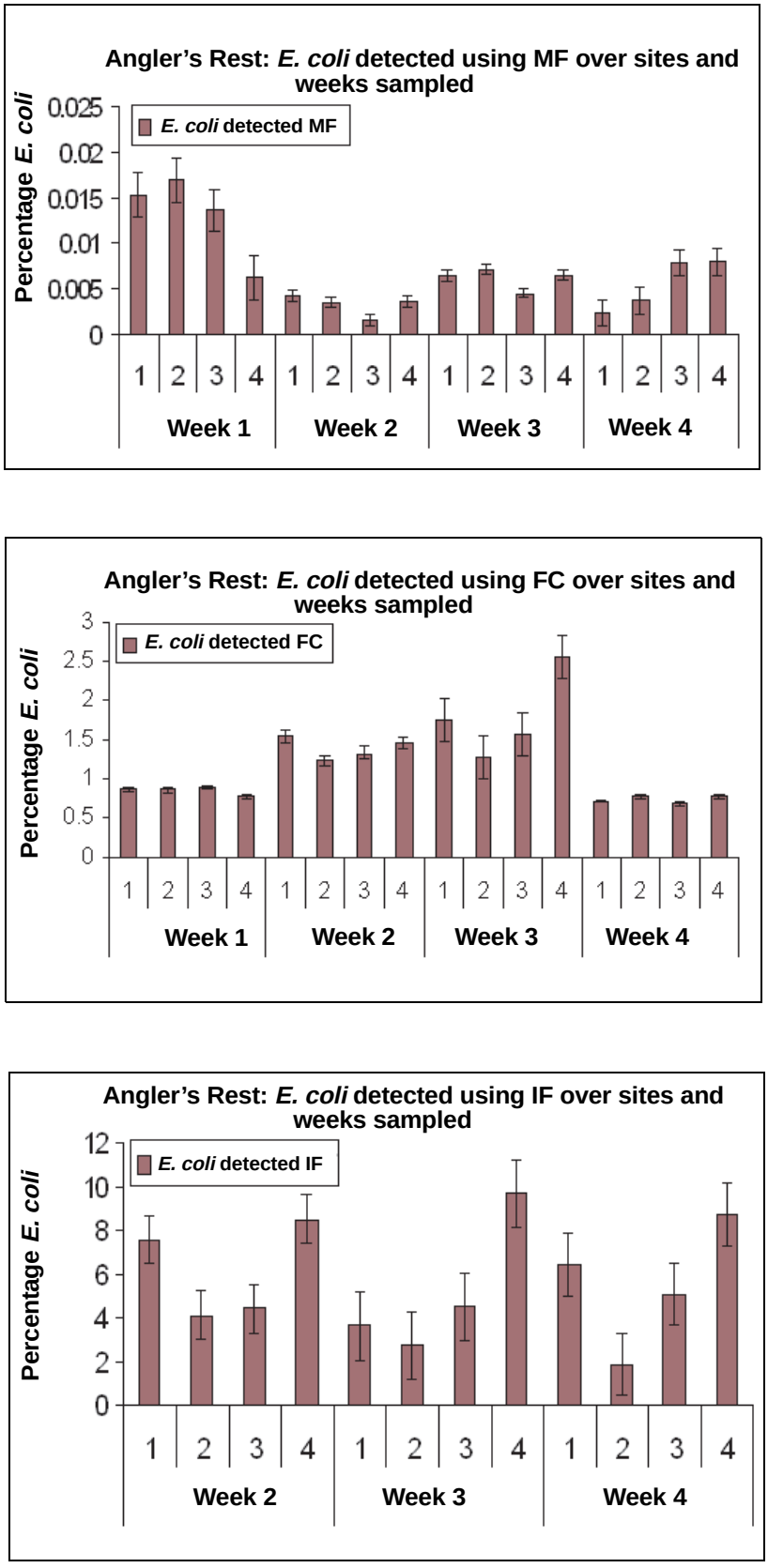



**Figure 4.13. The percentage of *Escherichia coli* detected at the Angler's Rest using each of the three detection methods over the sites and weeks sampled. (FC, flow cytometry; IF, immunofluorescence microscopy; MF, membrane filtration.)**

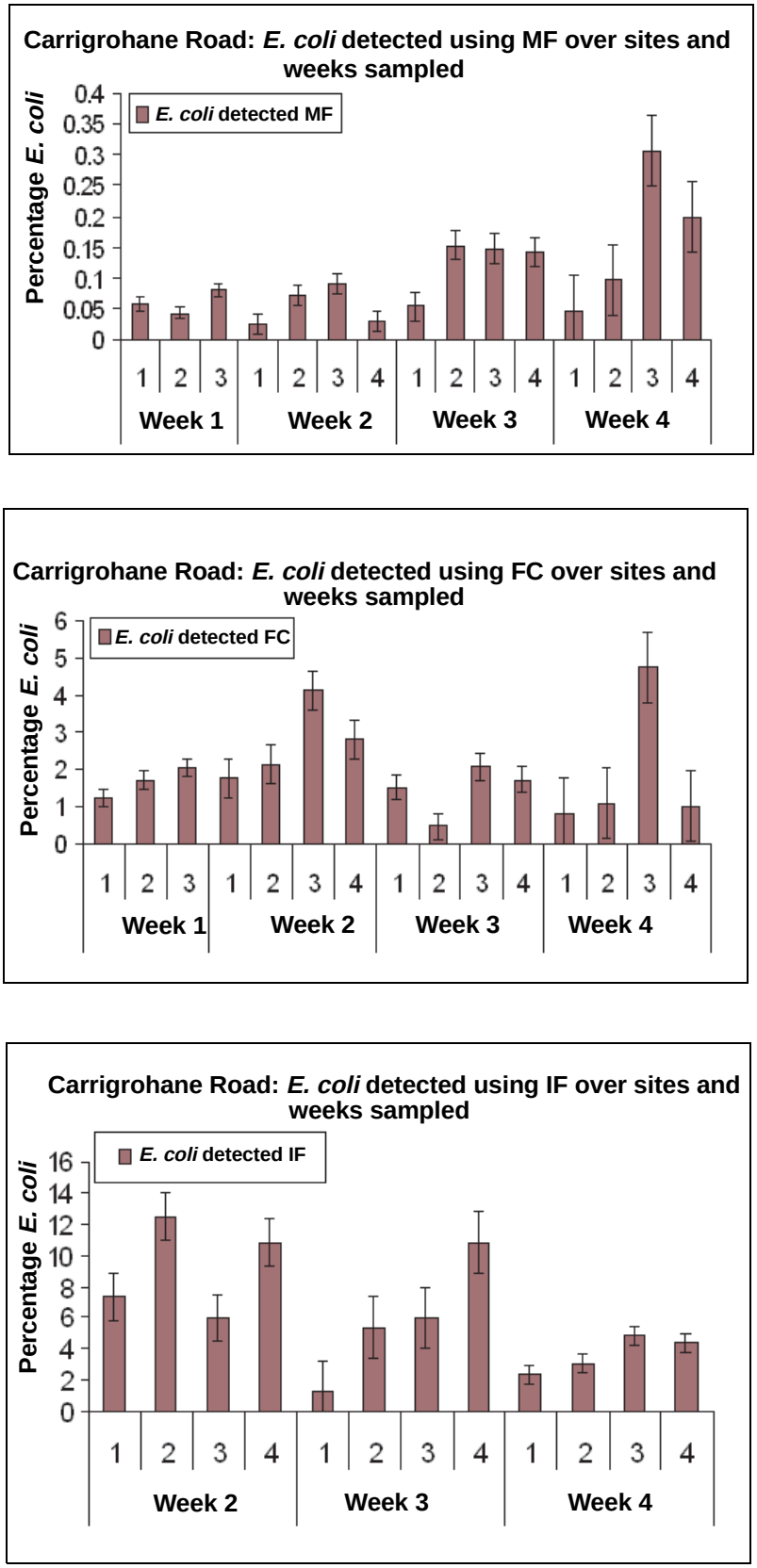



**Figure 4.14. The percentage of *Escherichia coli* detected at the Carrigrohane Road using each of the three detection methods over the sites and weeks sampled. (FC, flow cytometry; IF, immunofluorescence microscopy; MF, membrane filtration.)**

**Table 4.3. Washing steps experimented with using Kloehn filter pump showing the volumes of buffers and reagents used for washing and backwashing of bacteria in the Kloehn system. (*Backwash step of 1 ml was implemented here and was continued for the duration of experimentation; this lessened the occurrence of airlocks in the system. **Staining was carried out in this trial.)**

| Trial number | Step 1<br>Volume cells aspirated | Step 2<br>Volume PBS used for wash | Step 3<br>Volume backwashed for collection |
|---|---|---|---|
| **Trial 1** | 5 ml cells | 5 ml PBS | 5 ml |
| **Trial 2** | 2.5 ml cells | 5 ml PBS | 2 ml |
| **Trial 3** | 5 ml cells | 5 ml PBS | 3.5 ml |
| **Trial 4*** | 8 ml cells | 5 ml PBS | 2.5 ml |
| **Trial 5** | 2 ml cells | 5 ml PBS | 2 ml |
| **Trial 6** | 8 ml cells | 5 ml PBS | 2 ml |
| **Trial 7** | 8 ml cells | 5 ml PBS & Tween 80 | 2 ml |
| **Trial 8** | 8 ml cells | 5 ml PBS & Tween 80 | 2 ml |
| **Trial 9**** | 8 ml cells | 5 ml PBS & Tween 80 | 2 ml |

PBS, phosphate-buffered saline.

**Table 4.4. Flow rates used for each trial using the Kloehn filter pump; set speed volume of liquid/second for each trial.**

| Trial number | Intake | Output |
|---|---|---|
| **Trial 1** | 100 µl/s | 100 µl/s |
| **Trial 2** | 100 µl/s | 75 µl/s |
| **Trial 3** | 100 µl/s | 100 µl/s |
| **Trial 4** | 100 µl/s | 75 µl/s |
| **Trial 5** | 100 µl/s | 75 µl/s |
| **Trial 6** | 100 µl/s | 75 µl/s |
| **Trial 7** | 100 µl/s | 75 µl/s |
| **Trial 8** | 100 µl/s | 75 µl/s |
| **Trial 9** | 100 µl/s | 100 µl/s |

control of the motor and the input/output *via* a personal computer (Intellect II 1994).

Varied, known concentrations ($1 \times 10^4$ to $6.9 \times 10^8$ cells/ml) of suspended bacteria were pumped in through the chamber to determine the percentage recovery and to assess if the system could be successfully used to concentrate the bacteria for subsequent staining. Five replicates of suspended *E. coli*, at each of the concentrations, were filtered through the system at different flow rates using various volumes of liquid.

The volumes of liquid for washing bacteria were optimised throughout several trials (Tables 4.3 and 4.4). The bacteria in the chamber were washed initially using PBS, and then using Tween 80 at 1% concentration in PBS and later Tween 80 at 0.1%

concentration in PBS. The cells were then back-flushed through the system, using PBS or PBS/Tween 80, in order to remove the cells from the filter, and the filtrate was dispensed into a 20-ml sterile tube. The filters were removed from the chambers and examined to determine retention of *E. coli.* A wash and backwash step of PBS/distilled water was carried out to clean the lines between samples before and after each trial. A haemocytometer was used in order to enumerate the bacteria collected to estimate percentage recovery.

### *4.6.2 Antibody staining protocol*

A 1:25 dilution of antibody/isotype was added to the 2-ml cell suspension and mixed (Fig. 4.15). The cells were incubated in the dark at room temperature for 30 min. They were then pumped back into the machine, washed in 5 ml of 0.1% Tween/PBS and dispensed back out for examination using a fluorescent microscope. The millipore filtration chamber was encased in tinfoil to minimise light bleaching of the samples. Glycerol (1 ml) was added to the 5-ml sample and mixed gently in order to increase the viscosity of the suspension and reduce cell movement on the slides. A 10-µl volume of the cell/glycerol suspension was dropped onto a slide and a coverslip added. The slide was examined under an Olympus camera and a fluorescent microscope model BX51 TRF and five fields of view were photographed using Cell-F software from Olympus. Duplicate slides were examined for each sample.

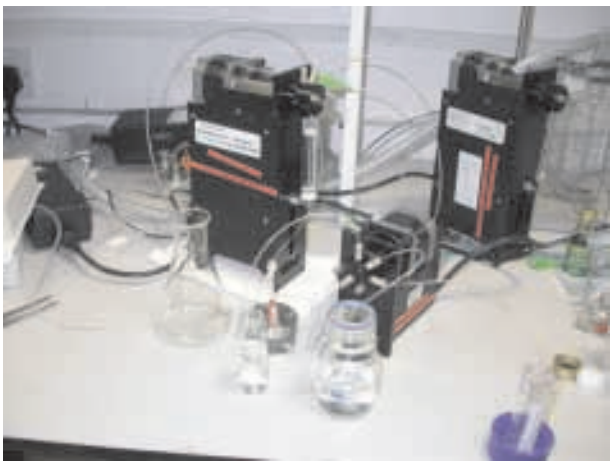


**Figure 4.15. Automated labelling system.**

### *4.6.3 Statistical analysis*

Statistical analysis was performed using Brodgar version 2.5.3. Data were compared using ANOVAs.

### *4.6.4 Results*

Initial trials did not concentrate the bacteria sufficiently and very low levels of bacteria were present in the samples. An average of $1.14 \times 10^4$ cells/ml were recovered from the $10^5$ samples, an 11.4% recovery rate, with 6.6% of the total population remaining on the filters (Fig. 4.16). The five replicates of the $1 \times 10^6$ dilution produced an average recovery of 54%, with approximately 8.4% of the total population of bacteria adhering to the filters.

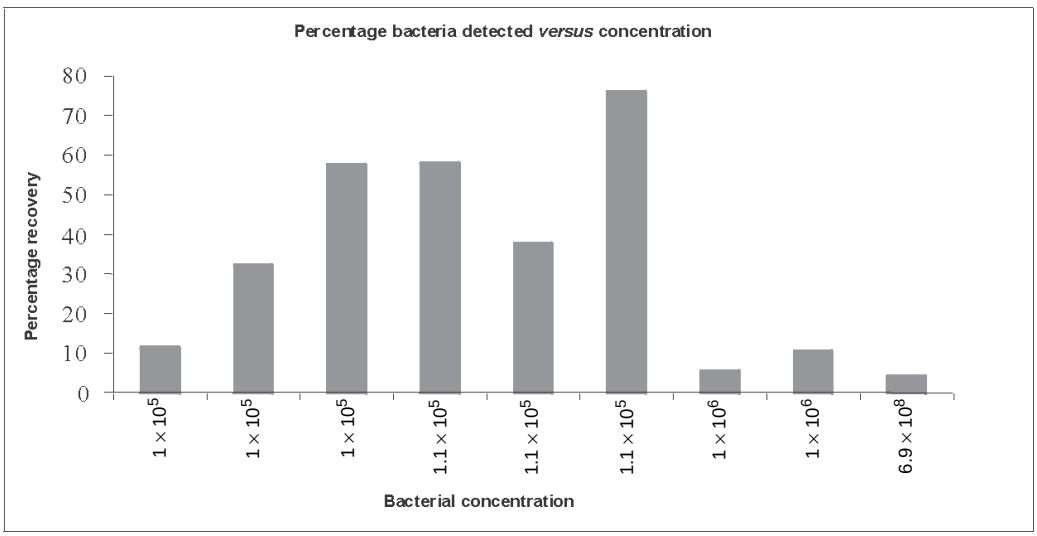



**Figure 4.16. Percentage of cells recovered at each of the bacterial concentrations.**

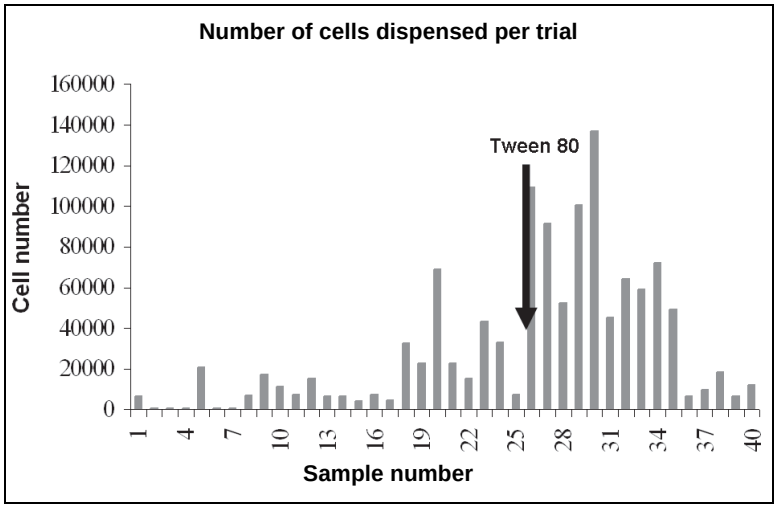



**Figure 4.17. The addition of 0.1% Tween to phosphate-buffered saline for the washing of bacteria – significant increase in the percentage recovery ($p < 0.01$, one-way ANOVA).**

Statistical analysis of the cell recovery data showed a significant increase in the numbers of cells recovered using Tween 80 (ANOVA: F = 5.84, df = 1, $p < 0.01$) (Fig. 4.17). There was also a significant difference in the proportions of bacteria obtained at the various bacterial concentrations utilised (ANOVA: F = 25.99, df = 3, $p < 0.0001$). There was no difference between the trials done on the Kloehn filter pump.

## 4.7 Discussion

Currently available microbiological-based methods for *E. coli* detection are inexpensive and reproducible; however, their primary disadvantages are that they are time consuming, requiring a lengthy incubation step, labour intensive, and underestimate the numbers of micro-organisms present (WHO, 2003). Immunological and molecular techniques often require specialist materials and are expensive. In this study optimisation was carried out on two commercially available antibodies for the detection of flow cytometry. Immunofluorescence microscopy and flow cytometry were utilised for the enumeration of antibody-tagged *E. coli*. There was no statistical difference between the staining proficiency of the two antibodies employed. The percentage staining was quite low, however, approximately 10% for the two antibodies. This demonstrates that more specific antibodies are required for the detection of these bacteria. *E. coli* cultures can be divided into more than 20 cell populations, with each of these populations representing a different stage of cell differentiation from each of the stages of growth, exponential through to stationary (Makinoshima *et al.*, 2003), and these different growth populations could explain the low staining percentage achieved by the fluorescent antibodies.

A fixation step is often included in immunological studies (Lopez-Amoros *et al.*, 1995; Marie *et al.*, 1996; Roth *et al.*, 1997); however, statistical analysis on the numbers of bacteria detected using the FITC-conjugated antibodies with and without fixation, showed no significant differences. This step was removed from the protocol, which reduced the time required for cell staining and detection by 20 min. A number of buffers and blocking agents were tested to determine their effects on the efficacy of antibody staining. Phosphate-buffered saline, bovine serum albumin and rabbit serum were all assessed at different concentrations and it was determined that a mixture of all three buffers and blocking agents provided the highest percentage of antibody staining and the lowest levels of non-specific binding.

Once optimal antibody staining concentrations and washing/blocking steps for the detection of *E. coli* were established, the antibody-staining technique was applied to environmental water samples. A comparison was made between flow cytometry,

immunofluorescence microscopy and membrane filtration, the government-approved standard method for the enumeration of coliform bacteria in bathing and drinking waters. Immunological staining of the bacteria was performed without the use of a cell pre-culturing step to increase bacterial numbers. Pre-culturing steps can drastically increase the time taken to determine the percentage of bacteria present in a sample. Using flow cytometry, removal of pre-culturing enabled enumeration of the proportion of *E. coli* present in an environmental sample to be performed in 1.5–2 h. Statistical analyses of the samples taken throughout this study showed a significant correlation between membrane filtration and flow cytometry. This result is noteworthy, as it suggests that flow cytometry could act as an alternative detection method for the enumeration of *E. coli* in environmental samples. In addition, the percentage of *E. coli* detected using flow cytometry was much higher than that of membrane filtration. This is because membrane filtration is capable of only detecting culturable bacteria present in water samples, whereas staining bacteria with antibodies is not dependent upon their viability and instead is based upon whether or not there is a suitable epitope (Makinoshima *et al.*, 2003; WHO, 2003). Immunofluorescence microscopy achieved the highest staining percentage of the three methods utilised for *E. coli* detection, and correlated significantly with flow cytometry. It was concluded that immunofluorescence microscopy was unsuitable for mixed bacterial work, as visual discernment of two bacterial populations from a slide was very difficult (Roszak and Colwell, 1987).

Of the two immunological methods utilised in the study, flow cytometry was found to be the most appropriate for the detection of specific bacteria in environmental samples. Porter *et al.* (1997) successfully utilised flow cytometry using immunological methods for the detection of bacteria in sewage and water samples.

Following determination of the suitability of flow cytometry for the detection of bacteria in water samples, an automated washing and staining protocol for bacterial cells was optimised using the Kloehn Intellect II. It was discovered that the addition of Tween 80, a non-ionic surfactant and emulsifier, gave a significant increase in the percentage recovery attained using the pump and filtration unit. Further study is required in this area to determine whether staining can be carried out within the millipore filtration chamber.

Direct antibody detection in conjunction with flow cytometry could eventually be used in addition to, or replace, the standard microbiological methods for the detection and enumeration of *E. coli* bacteria in drinking and bathing waters. The application of the staining and automated washing techniques optimised in this study to a miniaturised flow cytometry system would enable *in situ* analysis of *E. coli* and other bacterial and algal species of human and animal health significance in environmental waters.

# 5 Biofouling

## 5.1 Introduction

Microbial biofouling is caused by the undesirable adhesion and growth of bacteria, diatoms and fungi to/on surfaces submerged in fresh or saline water. It is of worldwide economic concern, costing industries billions of euro *per annum* in damage, repair and prevention. Industries with water-based processes, such as power generation stations and water desalination systems, are particularly prone to biofouling.

Most micro-organisms are capable of forming biofilms, as this is a universal mode of microbial life (Costerton *et al*., 1987), and it is a multistage process. Within minutes of immersing man-made surfaces (metals, plastics) in a non-sterile aqueous medium they adsorb a molecular 'conditioning' film, consisting of dissolved organic and inorganic material. Bacteria colonise the conditioned film within hours, certainly days, and within the weeks that follow unicellular algae/diatoms and cyanobacteria (blue–green algae) become established in the microbial community. Dense colonies of cells eventually arise due to rapid cell division, forming a microbial biofilm <500 µm in thickness.

If unchecked, marine and industrial biofouling will continue with visible colonisation by soft and hard macrofoulers, including algae, and invertebrates such as anemones, tunicates, soft corals, sponges, hydroids, tubeworms, mussels and barnacles. The specific fouling organisms that constitute a biofilm community depend on abiotic factors such as substratum, geographical location and calendar season, and biotic factors such as inter/intra-species competition and predation.

Global monitoring of the marine environment is increasing. The acquisition of continuous and long-term environmental parameter data is essential for managing freshwater, estuarine and coastal ecosystems that are vulnerable to pollution. Biofouling is the 'elephant in the room' that bedevils deployment of sophisticated microscale instruments in the aquatic environment. From a fabrication perspective, it is now possible to design highly reliable environmental monitoring instruments that will theoretically function autonomously for weeks/months. However, if their sample/data collection becomes unreliable within days because of biofouling, then that design effort is wasted. Much more work is needed before critical parameters can be remotely monitored on a long-term basis with no manual intervention.

Microfouling is particularly problematic for new, highly sophisticated and miniaturised technologies. Since first used in medical science, flow cytometry, a sensor technology that rapidly characterises optical properties of cells and cell components, is advancing research in numerous areas of ecology and evolutionary biology (Robinson and Gregori, 2007). By measuring the light emitted or scattered from cells and/or cell components using specific stains, a multitude of physical and genetic characteristics can be estimated simultaneously and rapidly processed.

The major difficulties associated with the field deployment of the miniaturised flow cytometer are 1) that when it is exposed to the marine environment clogging of the fluidic channels by suspended solid material may occur, and 2) following transportation of micro-organisms by convection, diffusion or sedimentation, biofilms will likely develop on material surfaces in direct contact with the external aqueous medium (Characklis and Marshall, 1990).

Clogging of channels has not attracted study previously, so the first objective of our investigations was to determine the magnitude of the problem at the fluidic channel scale and the flow rates envisaged for cytometer function. The second objective was to develop techniques for back-flushing to remove clogging material (probably combined with delivery of anti-microbial fluids to prevent biofilm development).

In theory, preventing the development of a biofilm should be as easy as controlling adhesion by the development of a surface on which bacteria cannot

settle. In reality, materials that completely inhibit microbial biofilm formation are non-existent despite the relatively large amount of research (Callow and Fletcher, 1994). However, one of the major objectives of the study that was achieved was to assess the fouling performance of a number of candidate fabrication materials under different conditions of flow and fouling pressure.

During the pilot phase of the programme, it was realised that no single material would be relatively immune to fouling. However, some published research suggests that surface microtopography might be important to microfouling intensity (Schumacher *et al.*, 2007). It was decided to take advantage of the microfabrication facilities of the Tyndall National Institute and a successful National Access Programme (NAP) proposal was written by the project participants. This allowed the deployment of replicated surfaces of various scales of microtopography to be deployed in the marine environment under different flow regimes. Samples have been collected, but analysis is in the early stages.

## 5.2 Qualitative and Quantitative Analysis of Fouling Cover in Lough Hyne

A problem for the field deployment of the miniaturised flow cytometer is that a fouling community is likely to accumulate on the external surface of the instrument. Biofilm formation and bacterial adhesion can occur within the first few hours of submergence in an aqueous environment. Larger organisms, such as fungi, diatoms, and the propagules of barnacles, mussels, polychaete worms, bryozoans and seaweed, will be likely to attach within the first few days of deployment.

### *5.2.1 Methods*

Tests were conducted at Lough Hyne nature reserve (Co. Cork) (Fig. 5.1) to consider the effect of biofouling on five different fabrication substrates (glass, PDMS, SU8, silicon, and poly(methylmethacrylate) (PMMA)) likely to form part of the external surfaces of the flow cytometer. A circular rig (Fig. 5.2) was designed to hold small pieces of each of the five materials that were all

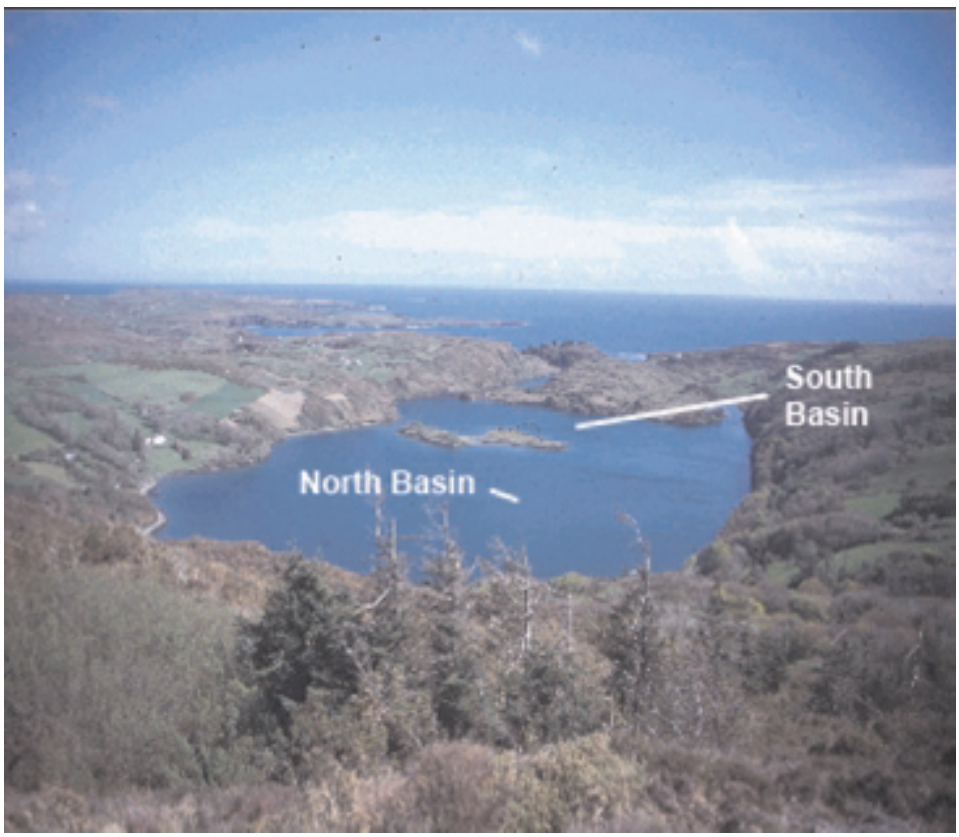



**Figure 5.1. Field test site at Lough Hyne, Co. Cork.**

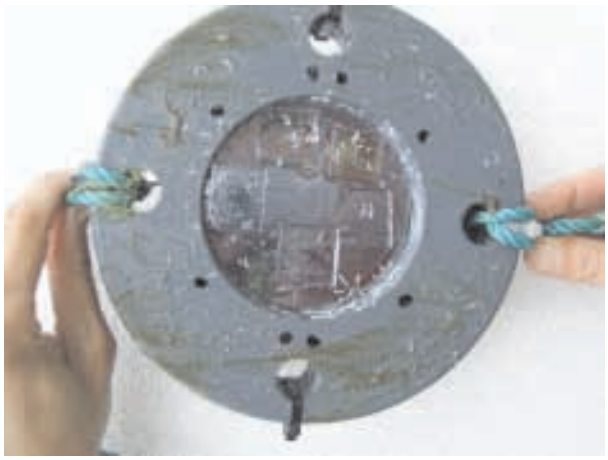


**Figure 5.2. Test rig holding silica disk with five test materials attached (recovered after 4-week exposure period).**

mounted on a single silica disk (120-mm diameter). Sixteen rigs were built in the ZEPS workshop, UCC, Co. Cork.

The test for biofouling was properly replicated (in triplicate) in terms of sample deployment. On 12 July 2007 the rigs were deployed in Lough Hyne in areas of relatively high (3 m/s) and low (5 cm/s) flow rates, and at two comparable depths (2 m and 10 m from the surface). After 1 month (13 August 2007) they were recovered, placed in scanning electron microscope (SEM) fixative *in situ* (see Fig. 5.3), stored and later prepared for investigation of fouling using an SEM. Light microscopy was employed to investigate larger fouling organisms and loosely attached material which had fallen into the storage vessels. The intention was to determine which of the materials tested was relatively less prone to fouling; the null hypothesis was that there would be no significant differences.

#### *5.2.1.1 Specimen preparation for scanning electron microscopy*

Materials were prepared for SEM analysis after appropriate primary fixation (materials were immersed in Karnovsky's fixative and then rinsed several times with PBS), post-fixation (materials were immersed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h and then rinsed with sodium cacodylate buffer for a minimum of 15 min) and dehydration (materials were immersed in an ethanol/water mixture of 70%, 95% and 100% for 10 min each, followed by chemical drying by hexamethyldisilazane (HMDS)). The procedure was as follows: 2 parts ethyl alcohol/1 part HMDS for 15 min, 1 part 100% ethyl alcohol/2 parts HMDS for 15 min, then two changes for 15 min each with 100% HMDS). The samples were then left to dry out in a desiccator overnight. For SEM analysis, samples were sputter-coated with gold (Agar Scientific Sputter Coater) and examined with a Hitachi S4000 SEM at 20 kV accelerating voltage.

#### *5.2.1.2 Quantitative image analysis*

Scanning electron microscope images of fouled materials were analysed for total percentage coverage using Image J software freely downloadable from http://rsbweb.nih.gov/ij/download.html. A total of six randomly chosen images (magnification: ×400) were analysed for particle coverage to represent each material at any given site (North Basin 2 m and 10 m, and South Basin 2 m and 10 m).

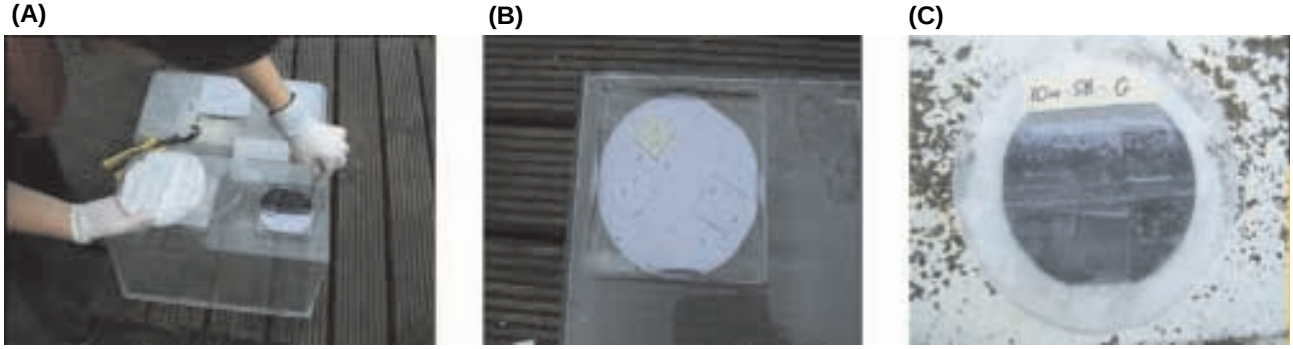



**Figure 5.3. Collection and fixation of samples. (A) Sample handling at Lough Hyne. (B) Primary fixation *in situ*. (C) Labelled and stored in sodium cacodylate buffer.**

Image J works by selecting features or particles of an image by thresholding, and then measures the number of red pixels. It is important to note that the term 'feature/particle' includes all inorganic and organic matter, so it includes bacteria, diatoms, ciliates, molluscs, annelids and phytodetritus (incorporating inorganic particles). Image J is then used to assess the proportion of an image covered by these various fouling materials.

#### *5.2.1.3 Statistical analysis*

Percentage coverage data cannot be distributed normally, so they were arcsine-transformed into degrees. All statistical procedures were conducted on the transformed data. Results were back-transformed into percentages where necessary. Mean coverages (with SD) were calculated. Comparisons were investigated employing ANOVA using the MINITAB package. One-way and two-way ANOVAs were used for preliminary exploration and comparisons; finally, a fully balanced nested ANOVA (three-way; Material × Site × Depth) was conducted on all data.

### *5.2.2 Results*

#### *5.2.2.1 Qualitative data*

Against expectation, materials were initially fouled, not with bacterial films, but by sticky phytodetritus. Presumably, such fragments of diatoms and dinoflagellates are present in the water column. Bacteria later appeared, as did a variety of diatoms (see Fig. 5.4).

Figures 5.5–5.8 show examples of low-power SEM pictures of different materials sampled in Lough Hyne at different depths and in different basins. At this magnification most fouling is visualised as diatoms, which are relatively large organisms, although fungi and accumulations of phytodetritus are also visible. It is evident that the materials exposed in the South Basin became much more fouled than those of the North Basin, and it is noticeable that diatomaceous fouling is also much more obvious in the South Basin.

Light microscopy was conducted on the macroscopic organisms that were associated with the materials when they were harvested at the end of the experiment. Examples of such organisms are shown in Fig. 5.9. No attempt was made to analyse these quantitatively as these organisms cannot be attributed to any individual test material, as it was loose material detached during *in-situ* chemical fixation and storage. Animals such as the periwinkles and harpacticoid copepods will have been attracted by the biofouling film and will browse upon it; the saddle oysters have attached to the materials just as they will to rock surfaces. Other species (e.g. echinoderm, barnacle larva) have probably just formed a temporary attachment to the surface.

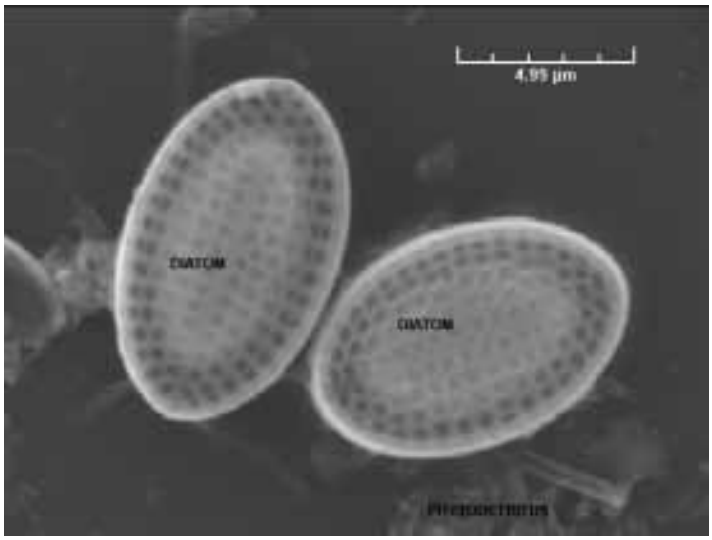



**Figure 5.4. Scanning electron microscopy photograph of surface of poly(methylmethacrylate) (PMMA) test material (×6000 magnification).**

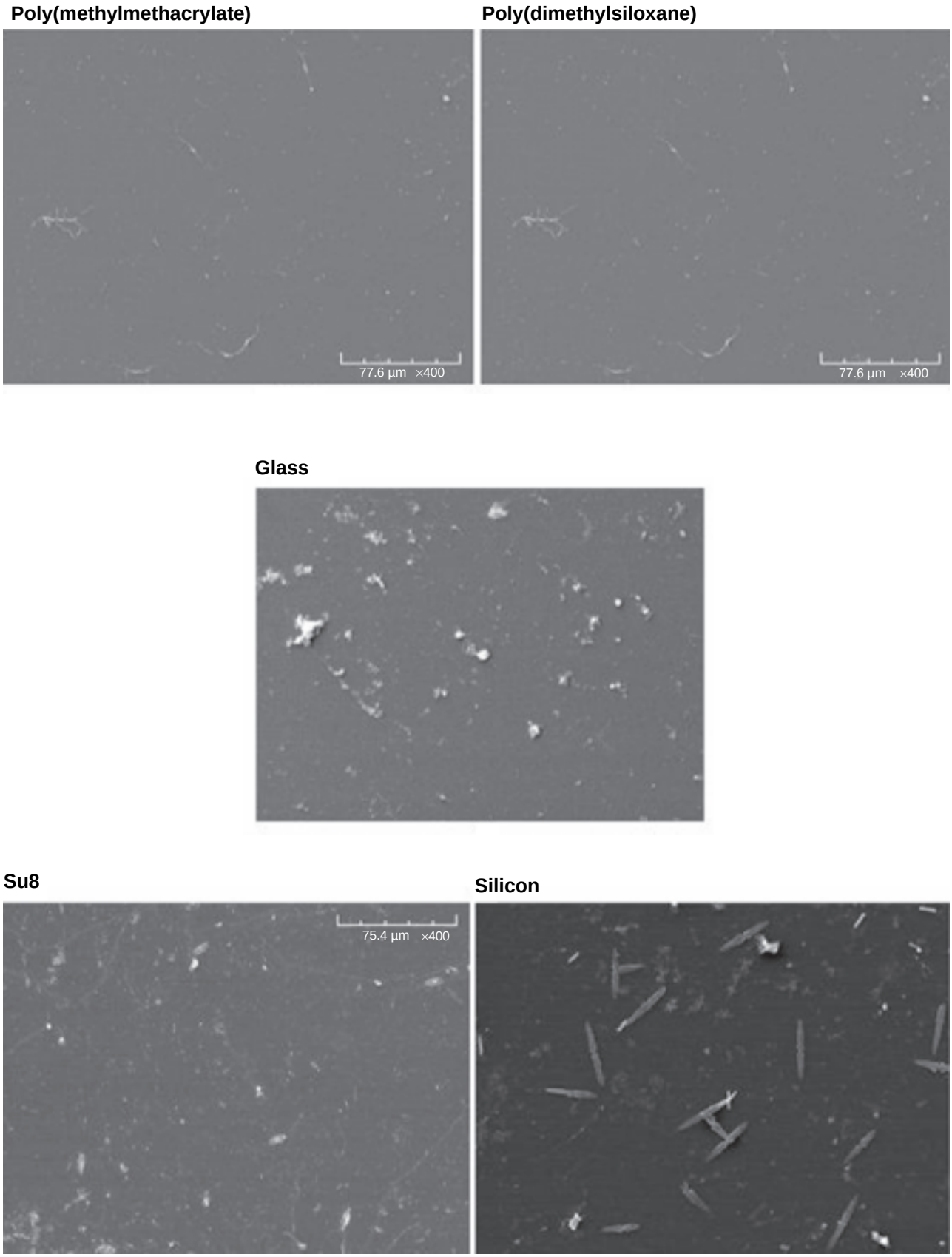
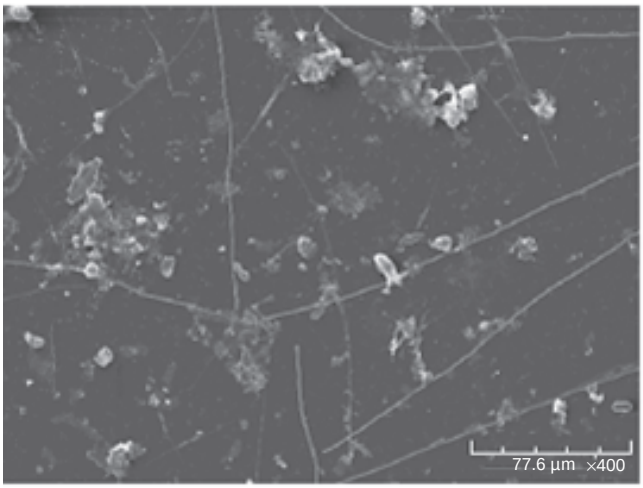
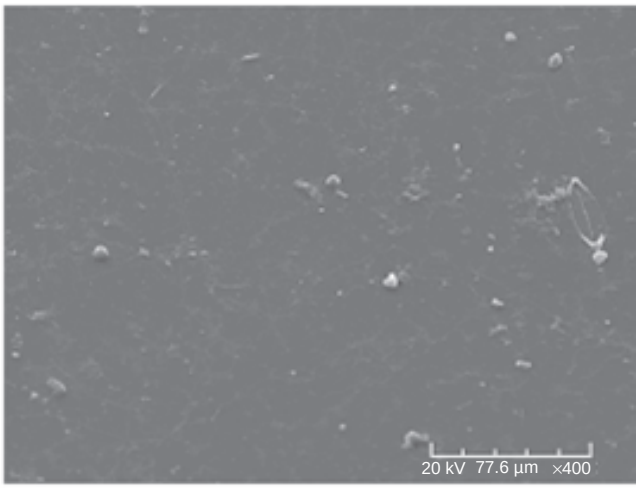



**Figure 5.5. Summary of low magnification images (×400) of different materials exposed to the environment in Lough Hyne. North Basin, 2 m depth.**

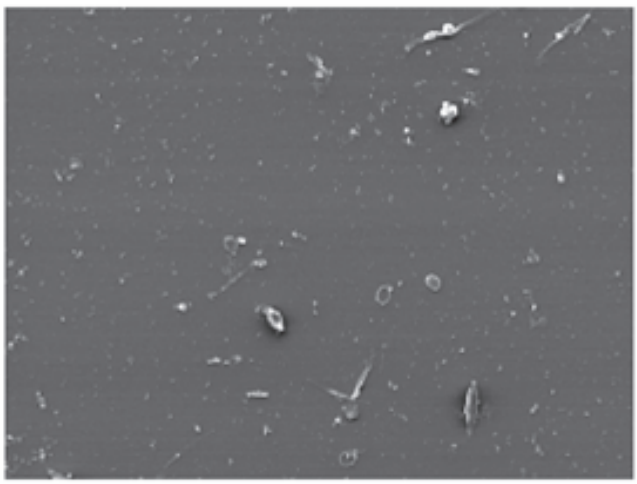
**Poly(methylmethacrylate)**



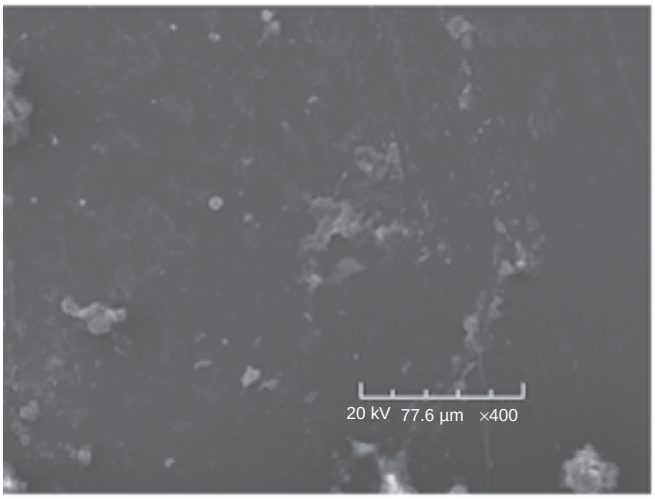

**Poly(dimethylsiloxane)**




**Glass**



**Su8**




**Silicon**

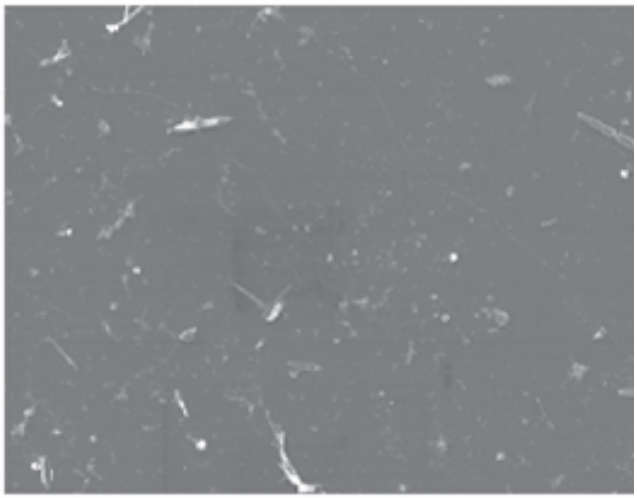


**Figure 5.6. Summary of low magnification images (×400) of different materials exposed to the environment in Lough Hyne. North Basin, 10 m depth.**

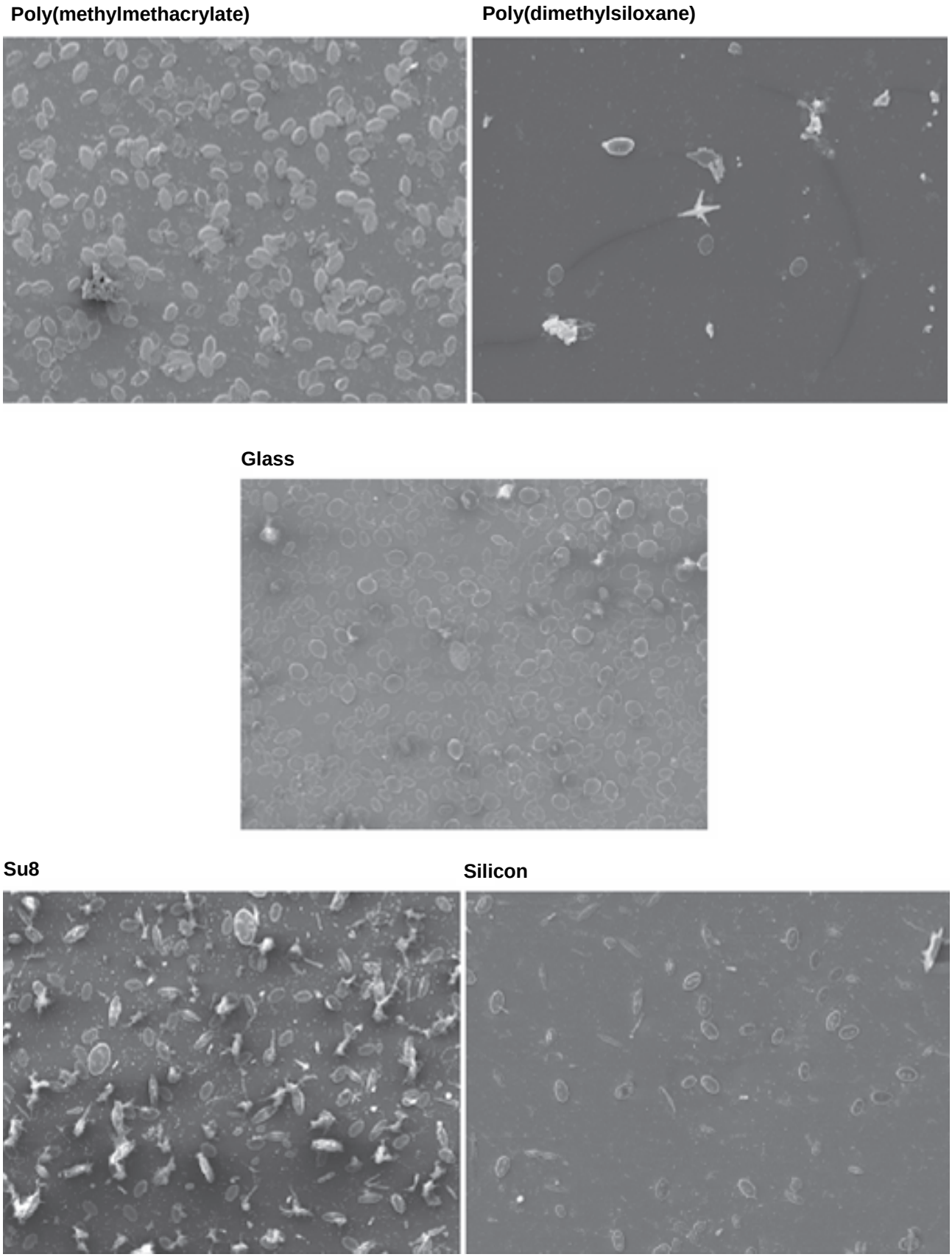
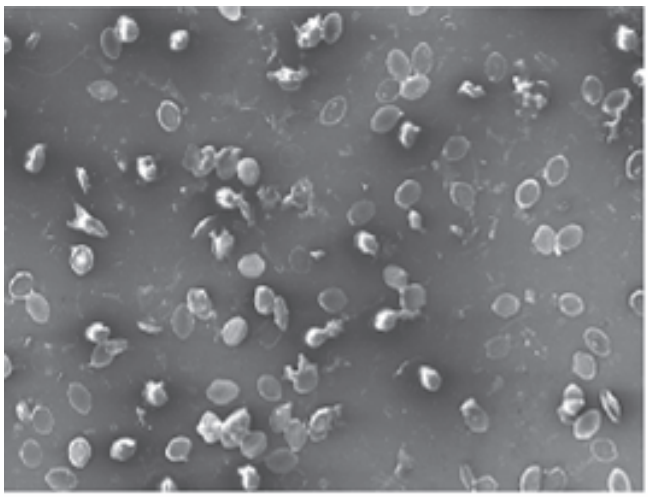
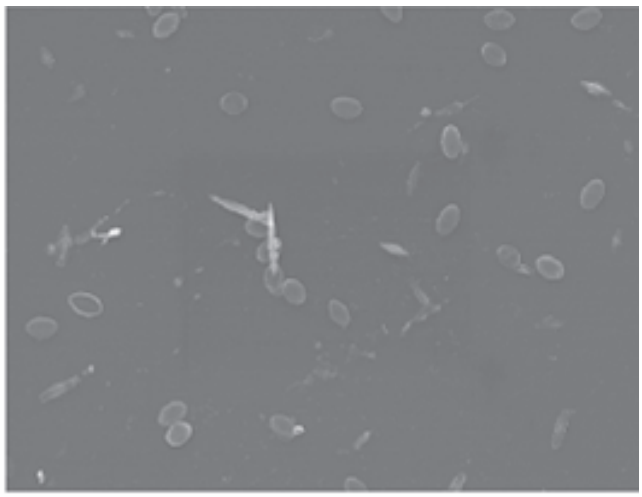



**Figure 5.7. Summary of low magnification images (×400) of different materials exposed to the environment in Lough Hyne. South Basin, 2 m depth.**

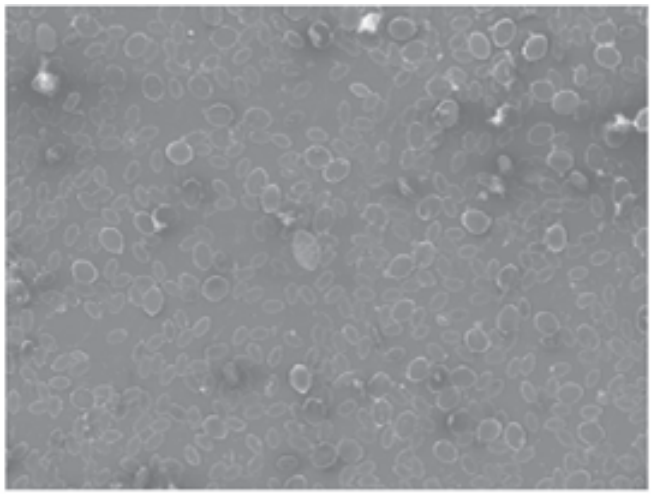
**Poly(methylmethacrylate)**



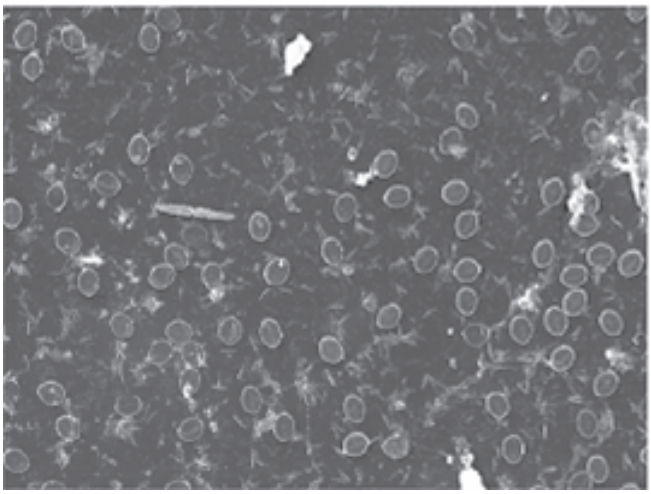
**Poly(dimethylsiloxane)**



**Glass**



**Su8**



**Silicon**

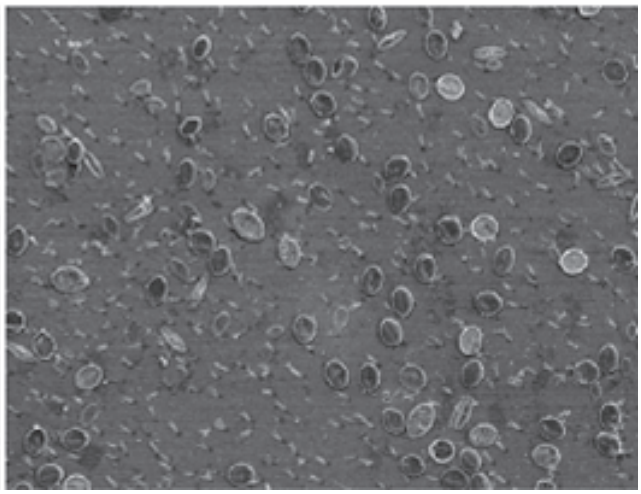
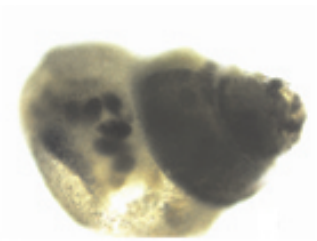
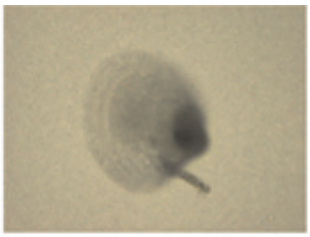


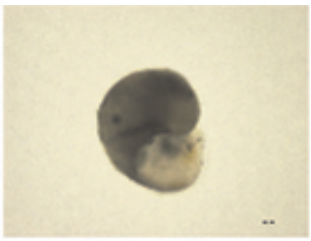
**Figure 5.8. Summary of low magnification images (×400) of different materials exposed to the environment in Lough Hyne. South Basin, 10 m depth.**



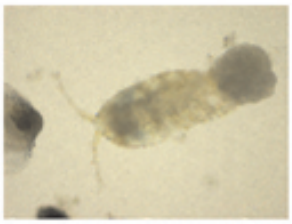
**Mollusca: Gastropod: Periwinkle – *Littorina littorea* (juvenile)**



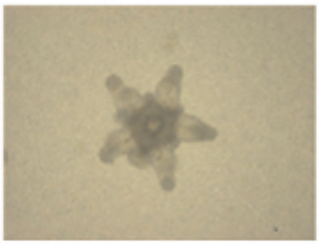
**Mollusca: Bivalve: Saddle oyster – *Anomia ephippium***



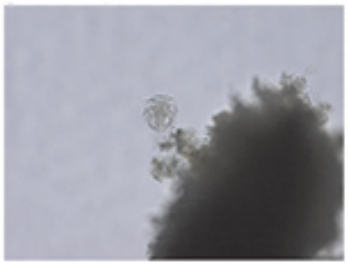
**Mollusca: Gastropod: Flat periwinkle – *Littorina mariae* (juvenile)**



**Crustacea: Copepoda: Harpacticoida (adult female with egg mass)**



**Echinoderm: (disc diameter 450 µm). Fully formed, detached juvenile disc.**



**Crustacean: Cirripedia: barnicle nauplius larva**

**Figure 5.9. Examples of macroscopic animals associated with fouling of materials deployed in Lough Hyne.**

#### *5.2.2.2 Quantitative data*

The basic coverage data are presented in Table 5.1. All material samples showed some fouling (at least 4%), and some showed mean coverage as high as 34% despite a relatively short exposure period of 28 days.

#### *5.2.2.3 ANOVA investigation*

Much detailed information was extracted by one-way and two-way ANOVAs, demonstrating significant differences (often highly significant differences) amongst materials and between basins and depths. Here only the results of the overall fully balanced (three-way) ANOVA are given (Table 5.2).

Basically this analysis means that, overall, there is no consistent pattern of fouling intensity of different materials across the two basins and two depths. However, there are highly significant differences ($p = 0.001$) in fouling rates between the two basins, which have markedly different flow regimes and water residence times (South Basin: high flow rate, residence time in hours/days; North Basin: low flow rate and high residence times (<40–50 days)). From inspection of Table 5.1 it may be seen that, in the great majority of cases (19 out of 20; exception – Si at 10 m), when comparing the same depths, fouling was more intense in the South Basin. This analysis also reveals extremely significant effects of depth ($p < 0.0005$) on fouling. However, detailed analysis (see Table 5.1) reveals highly significant differences between depths for many materials, but not in a consistent direction.

### *5.2.3 Discussion*

From a materials point of view the data are straightforward: all could be incorporated in a flow cytometer design without significant advantage/ disadvantage (despite differences in material roughness and contact angle). From Table 5.1 it may be seen that PDMS generally showed the least fouling, but the advantage is not statistically significant.

Operationally, the data are interesting because they dramatically reveal the difference in fouling pressure between deployment sites, even within a single body of water. In Lough Hyne, the South Basin samples were much more prone to fouling than in the North Basin. The reasons are multifactorial. Flow rates are higher in the South Basin and residence times much lower. This means that boundary layer phenomena will differ between the two sites, but there will also be differences in the quantity of propagules and possible differences in nutrients/micronutrients. From a design and operational viewpoint, any antifouling approach will have to be effective immediately against a wide range of fouling pressures.

**Table 5.1. Summary of biofouling coverage of materials tested at Lough Hyne, 12 July 2007.**

| Material + Site + Depth | Mean coverage (%) | SD (%) |
|---|---|---|
| **Poly(dimethylsiloxane)** | | |
| **North Basin 2 m** | 4.06 | 0.325 |
| **North Basin 10 m** | 12.78 | 0.451 |
| **South Basin 2 m** | 8.35 | 0.081 |
| **South Basin 10 m** | 15.90 | 0.660 |
| **Poly(methylmethacrylate)** | | |
| **North Basin 2 m** | 7.29 | 0.023 |
| **North Basin 10 m** | 5.60 | 0.129 |
| **South Basin 2 m** | 33.61 | 0.381 |
| **South Basin 10 m** | 13.88 | 1.704 |
| **Glass** | | |
| **North Basin 2 m** | 4.42 | 0.105 |
| **North Basin 10 m** | 6.54 | 1.309 |
| **South Basin 2 m** | 21.32 | 0.179 |
| **South Basin 10 m** | 20.61 | 0.093 |
| **SU8 (photoresist polymer)** | | |
| **North Basin 2 m** | 11.53 | 0.312 |
| **North Basin 10 m** | 5.93 | 0.401 |
| **South Basin 2 m** | 13.18 | 0.073 |
| **South Basin 10 m** | 29.88 | 0.180 |
| **Silicon** | | |
| **North Basin 2 m** | 7.16 | 0.176 |
| **North Basin 10 m** | 16.45 | 0.624 |
| **South Basin 2 m** | 12.34 | 0.100 |
| **South Basin 10 m** | 13.56 | 0.070 |

**Table 5.2. Fully nested analysis of variance (takes into account basin, materials, depth).**

| Source of variance | df | SS | MS | F value | *p* |
|---|---|---|---|---|---|
| **Basin** | 1 | 2619.3092 | 2619.3092 | 25.6420 | 0.0010 |
| **Materials** | 8 | 817.7639 | 102.2205 | 0.5360 | 0.806(NS) |
| **Depths** | 10 | 1907.1834 | 190.7183 | 15.7680 | <0.0005 |

MS, mean squares; SS, sum of squares.

## 5.3 Quantitative Analysis of Suspended Solids and Susceptibility of Fluidics to Clogging during Field Deployment

A potential problem for the field deployment of the miniaturised flow cytometer is that it will be exposed to suspended solid material (inorganic/organic) that can potentially cause clogging of the inlet filter and/or fluidic channels.

### *5.3.1 Methods*

Tests were conducted to determine whether the fluidic channel (diameter: 750 µm) of an operational single-channel peristaltic pump was susceptible to clogging when exposed to sea water, riverine water and three concentrations of *Chlorella vulgaris* and *Saccharomyces cerevisiae* cells in suspension. To rule out flow rate as a factor that might cause a blockage, five different flow rates were used throughout the experiment.

#### *5.3.1.1 Qualitative analysis of a pump's clogging susceptibility to natural sea water and river water.*

Natural sea water (obtained from the main storage tank of the Aquaculture & Fisheries Development Centre, UCC) and river water (sourced from the River Lee) were constantly pumped from large vessels through 60 cm of clear plastic tubing for at least 10 h under supervision. The pump's tubes were inspected for blockages by debris at hourly intervals.

The inevitable settlement of particles within the sea-water and river-water vessels was counteracted by the use of a magnetic stirrer. Particle size distributions/ densities within sea water and river water were obtained by a Z2™ COULTER COUNTER® Cell and Particle Counter and can be seen in Table 5.3.

#### *5.3.1.2 Quantitative analysis of clogging susceptibility to Chlorella vulgaris and Saccharomyces cerevisiae*

The mean spherical diameter of *Chlorella vulgaris* (a single-celled, freshwater alga) determined by light microscopy was 5.5 µm. These are relatively small cells, but are known to clump together and reach very high concentrations. *Saccharomyces cerevisiae* cells, commonly known as baker's yeast, are larger; the mean cell size observed was 10 µm (length) and 5 µm (width).

**Table 5.3. Summary of Z2™ COULTER COUNTER® particle counts of sea-water and river-water samples.**

| Particle size | Sea water | River water |
|---|---|---|
| **0–10 µm** | 7,946,512 | 4,837,424 |
| **10.0–20.0 µm** | 752 | 328 |
| **20.0–30.0 µm** | 40 | 16 |
| **30.0–40.0 µm** | 8 | 0 |

Predetermined nominal concentrations of *C. vulgaris* cells ($2 \times 10^4$, $2.2 \times 10^5$ and $1.5 \times 10^6$ cells/ml) were pumped through 60 cm of a single fluidic channel of constant diameter (750 µm), at five different flow rates, 25,756, 54,256, 82,860, 109,385 and 132,812 µl/min, for at least 1 h. Cell density of a suspension was determined using a haemocytometer and means of triplicate measurements were used as representative values. Cell density input (i.e. concentration in upstream vessel) and output (flow from pump tubing) were estimated three times for each cell concentration and pump flow rate used.

*S. cerevisiae* cells were also used to assess possible clogging because of their larger cell size, oval shape and ability to reach far greater concentrations. The experimental procedure was repeated as outlined above for *Chlorella*.

### *5.3.2 Results*

Sea-water and river-water particle characteristics are given in Table 5.3. Empirical observations showed that no clogging occurred. No large visible debris was sighted in the tubing during any of these trials.

For the experiments with *Chlorella* and yeast cultures, ANOVA comparisons between the means of cell input and output concentrations for every flow rate and concentration combination were made to test whether concentrations were affected by passage through the pump (see Table 5.4 for data). In all cases there were no significant differences between inflow and outflow concentrations. No clogging was observed in any case, even though *Chlorella* cell aggregates

**Table 5.4. Comparison of inflow and outflow concentrations of *Chlorella* and yeast counts at five flow rates and three concentrations.**

| Nominal concentration level cells/ml | Inflow counts (cells/ml) | | | | | Outflow counts (cells/ml) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Flow rate (µl/min):** | **25,756** | **54,256** | **82,860** | **109,385** | **132,812** | **25,756** | **54,256** | **82,860** | **109,385** | **132,812** |
| ***Chlorella*** | | | | | | | | | | |
| **×10$^4$** | 25,000 | 27,000 | 20,000 | 20,000 | 22,500 | 20,000 | 20,000 | 17,500 | 17,500 | 27,500 |
| | 20,000 | 22,500 | 20,000 | 27,500 | 22,500 | 22,500 | 17,500 | 22,500 | 17,500 | 25,000 |
| | 20,000 | 12,500 | 20,000 | 17,500 | 25,000 | 17,500 | 17,500 | 20,000 | 22,500 | 17,500 |
| **×10$^5$** | 275,000 | 177,500 | 255,000 | 275,000 | 200,000 | 275,000 | 177,500 | 255,000 | 225,000 | 185,000 |
| | 185,000 | 275,000 | 275,000 | 232,500 | 255,000 | 250,000 | 262,500 | 287,500 | 247,500 | 265,000 |
| | 210,000 | 235,000 | 237,500 | 215,000 | 262,500 | 237,500 | 275,000 | 255,000 | 230,000 | 295,000 |
| **×10$^6$** | 1,590,000 | 1,545,000 | 1,482,000 | 1,537,500 | 1,540,000 | 1,505,000 | 1,490,000 | 1,510,000 | 1,480,000 | 1,530,000 |
| | 1,497,500 | 1,605,000 | 1,582,500 | 1,570,000 | 1,650,000 | 1,505,000 | 1,397,500 | 1,565,000 | 1,547,500 | 1,525,000 |
| | 1,560,000 | 1,457,500 | 1,500,500 | 1,540,000 | 1,487,500 | 1,547,500 | 1,522,500 | 1,482,500 | 1,605,000 | 1,480,000 |
| **Yeast** | | | | | | | | | | |
| **×10$^4$** | 15,000 | 17,500 | 17,500 | 22,500 | 25,000 | 22,500 | 15,000 | 15,000 | 15,000 | 20,000 |
| | 17,500 | 27,500 | 27,500 | 25,000 | 20,000 | 15,000 | 22,500 | 20,000 | 22,500 | 20,000 |
| | 25,000 | 20,000 | 12,500 | 20,000 | 22,500 | 15,000 | 20,000 | 17,500 | 27,500 | 20,000 |
| **×10$^5$** | 237,500 | 275,000 | 282,500 | 287,500 | 305,000 | 255,000 | 215,000 | 215,000 | 287,500 | 295,000 |
| | 347,500 | 295,000 | 277,500 | 275,000 | 252,500 | 217,500 | 295,000 | 275,000 | 305,000 | 252,500 |
| | 255,000 | 285,000 | 267,500 | 255,000 | 260,000 | 262,500 | 252,500 | 252,500 | 252,500 | 260,000 |
| **×10$^6$** | 2,810,000 | 2,867,500 | 2,715,000 | 2,830,000 | 2,922,500 | 2,515,000 | 1,865,000 | 2,677,500 | 2,975,000 | 3,105,000 |
| | 3,282,500 | 2,875,000 | 3,245,000 | 2,617,500 | 2,472,500 | 2,152,500 | 2,642,500 | 2,877,500 | 3,095,000 | 2,735,000 |
| | 2,685,000 | 2,685,000 | 2,992,500 | 2,867,500 | 2,730,000 | 2,370,000 | 2,387,500 | 3,195,000 | 2,812,500 | 2,422,500 |

(maximum four cells) were observed in the haemocytometer counting chamber.

### *5.3.3 Discussion*

The clogging trials have been entirely satisfactory; no clogging was seen either with natural waters or pure cultures even over periods of many hours and at high particle concentrations ($<3 \times 10^6$ cells/ml). With the tubing diameter used, there was no indication that the pump/tubing combination selectively removed either fluid or particles. Taken together, these data indicate that the cytometer design should not be prone to frequent blockages, or require pre-filtering.

# 6 Overall Conclusions

Progress towards the development of an alignment-free optofluidic system for cytometry has been described.

With regard to the specific objectives of the research programme:

1. *Design, fabrication and testing of a lab-based demonstration/proof-of-principle cellular analysis unit showing required sensitivity of proposed autonomous system*

   The development of a fully functioning prototype cytometer was not achieved with only a partial integration of components realised. Issues with the packaging and integration of optical components were encountered which impacted on the overall progress of the project including work packages of partners in ZEPS, UCC. While individual particle counts have yet to be resolved, a functioning detector array was demonstrated which could ultimately be used for particle counting and other chemical and biological applications. Heterogeneous integration of highly sensitive, space and power-efficient components (APDs and microLEDs) with microfluidic channels was investigated using specialist flip-chip alignment and attachment methods. Additional outputs of the research programme include the design of innovative approaches to the heterogeneous integration of key optical elements, which provides the foundations for the demonstration of the system concept without the need for laser drilling of components. The preferred embodiment of the optical module design includes a light source and detection element pre-aligned to a microfluidic channel. Key actions required to determine whether or not the proposed approach will achieve sensitive particle detection have been determined. These include wavelength-specific testing of illumination output of fully packaged microLEDs as a function of operating power. Testing of dark counts and noise background of fully packaged APDs is necessary to ensure that the arrays are functional after packaging. Wavelength-specific detection of scatter and fluorescence is most likely required for microparticles and cells in the microfluidic interrogation zone. The detection of particle scatter/fluorescence using a commercially available APD system could be used to establish the relative sensitivity of the Tyndall module.

   Much has been learned throughout the course of the project about the development of an integrated cytometry system. The establishment of new groups specialising in the area of heterogeneous integration and optical packaging along with the acquisition of specialist equipment has added to the capabilities of the team to achieve original project goals. Future iterations of the system will use a different approach to the packaging of optical components without the need for laser processing. In addition, more powerful and tailored optical sources have recently become available for testing which will add capabilities in the area of particle detection. Due consideration of the need for integration of optical filters will also be explored and some initial discussions with external companies working in the space have begun.

2. *Selection and implementation of an immunological technique for identification of cellular species of interest, e.g.* E. coli*, with subsequent benchmarking against standard reference methods*

   The development of an immunolabelling protocol for *E. coli* detection was explored for molecular-level biological recognition. Conventional IF microscopy and bench-top flow cytometry were utilised for the enumeration of antibody-tagged *E. coli* in water samples. Optimisation was carried out using two commercially available antibodies but no statistical difference in staining proficiency was found. The percentage staining was relatively low for both antibodies at approximately 10%.

This demonstrates that more specific antibodies are required for the detection of these bacteria. Of the two immunological methods utilised, flow cytometry was found to be the most appropriate for the detection of specific bacteria in environmental samples tested.

3. *Investigatation of enhancing the period of use of the proposed system without manual intervention.*

   Assessment of potential risks to system operation from clogging and biofouling included the use of fresh and marine samples with varying degrees of particulate loading.

   The magnitude of clogging at the microfluidic scale with flow rates envisaged for cytometer function was investigated. Results of initial flow trials performed over several hours showed no clogging by natural water or pure cell culture samples, even at high particle concentrations ($<3 \times 10^6$ cells/ml). An assessment of the fouling performance of typical fabrication materials remotely deployed under different conditions of flow and fouling pressure suggests that all could be incorporated in a flow cytometer design without significant advantage/disadvantage. Results to date indicate that the proposed cytometer design should not be prone to frequent blockages, or require pre-filtering.

# 7 Future Research

The major research needs associated with the creation of miniaturised optofluidic cytometry systems such as those described in this report are related to four key issues.

1. *Light source*

   The availability of small light sources with wavelengths that match the excitation of commercial fluorescently labelled antibodies is a key issue for system-level integration. The power density of the light source needs to be tightly controlled along with the angle of dispersion of the emitted light. The microLED sources evaluated in this project gave a reasonably pseudo-collimated beam; however, further work is needed to establish if the power output is sufficient for cytometry applications. Alternative illumination sources such as vertical-cavity surface-emitting lasers (VCSELs) may be required to create an output of sufficient intensity to allow detection of single cells through scatter and fluorescence measurements.

2. *Detector and optics*

   Light from cells/particles generated by fluorescence or scatter needs to be directed into one or more detectors. The stimulation light also needs to be filtered to minimise background contributions. In this instance, the size of the active areas of the detector array matches that of the fluidic channel. However, interactions between the devices in the array were not evaluated and in any further programme optimised device spacing will need to be defined.

3. *Alignment issues*

   The light sources were manually aligned to the fluidic channel. This introduced a certain amount of error through the placement of the devices. The next iteration of the system must address this by aligning the source to the channel during the fabrication step using optically transparent substrates such as glass. This would allow the production of alignment-free systems and reduce associated alignment errors.

4. *Electronics and packaging*

   It was not possible to introduce an optical filter into the system without an additional lithography step. The lack of optical filtering capability resulted in high noise backgrounds. The challenge is to integrate optical filters onto the detector array during the fabrication stage to allow selective detection of the emitted/reflected light. More work is needed to tailor the detector devices to the specific requirements of the cytometry application.

The clogging trials (which revealed no problems whatsoever in the short-term study) need to be pursued over longer periods to monitor the development of biofilms within the fluidic channels. If the NAP proposal relating to the influence of surface architectures on fouling intensity demonstrates significant results, the findings will be used to support a proposal to Science Foundation Ireland (SFI) for further funding. Back-flushing and antifouling protocols for the fluidic channels need to be developed. The use of antifouling coatings on external surfaces of the flow cytometer need to be considered, since one of the findings of the study was that of substantial fouling by diatoms (that change the environment around the instrument) occurring in environments with high flow rate and high light intensity.

In relation to the immunological investigations research needs, the optimisation of antibodies including the development of new antibodies would facilitate the specificity of the flow cytometry method. In addition the method could then be applied to screen for other water-borne organisms of interest, e.g. algal monitoring, parasites/pathogens of humans, fish and shellfish. The method could be modified to screen algal species of interest and could be applied to monitoring and research programmes where, for example, pathogen levels in the water column need to be

screened and levels detected to develop a warning system for various diseases of aquatic organisms.

The continued need for a system such as that originally proposed is evident from the continued rate of research-level publications and commercial interest in this area. There is a clear market niche for cell monitoring with the majority of commercial cell-counting systems confined to lab use and not inherently portable in nature. For optical detection capabilities, a bench-top apparatus with delicate optical alignment requirements is still the standard in the majority of research laboratories with only a few examples employing truly miniaturised integrated optical components. The integration of optics with fluidic networks is still an area open to exploitation and is envisioned as a key enabling technology over the coming years (Huh *et al.*, 2005). In this regard the innovative approaches to the integration of optical elements with fluidic networks that have been achieved thus far in Tyndall provide considerable foundations for realisation of the proposed cytometry unit. The approach to design, packaging and assembly of key optical components within microfluidic devices has, in itself, been an advance beyond the current state of the art.

The potential development of new intellectual property (IP) is high as a result of ideas that came about from this project. Funding for the further development of the optofluidic platform technology is being sought through other programmes including from Enterprise Ireland.

## 7.1 Funding

This project attempted to combine multiple micro-technologies for the generation of a new platform for marine analysis. The development of complex technology-orientated analysis platforms requires a customised systems-level approach to design and fabrication. Further work is needed to achieve a fully integrated system. Subsequent joint funding applications have been submitted incorporating the proposed cytometry system to national agencies, specifically Science Foundation Ireland (Strategic Research Cluster Call), Enterprise Ireland (CFTD and POC). A resubmission in the Proof-of-Concept Call is currently under evaluation for funding and a proposal to develop a similar system is also under way with an international funding agency. Several Irish start-up companies have also expressed an interest in the commercialisation of the outputs of this research.

Many of the key challenges in addressing future and emerging issues in health, the environment and energy will require multidisciplinary research of increasingly complex engineered systems. Tyndall's strategy has three themes in the area of sensor development: health, environment and data transmission. The Microsystems Centre at Tyndall, in association with a range of collaborators in engineering, computer science and bioscience, engages in multidisciplinary research, education and outreach with a focus on the application of information and communications technology (ICT) to the design, operation and maintenance of complex engineered systems. Examples include building energy management, remote environmental monitoring, point-of-care (POC) diagnostics and targeted *in-vivo* theranostics. Many of the resulting research programmes are based on integrated approaches to the generation, acquisition, storage, dissemination and manipulation of information. These synergistic collaborations seek to develop ICT-enabled platforms and systems that seamlessly connect people with their physical and information environments.

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# Glossary

**ANOVA** Analysis of variance

**APD** Avalanche photodiode

**ATCC** American Type Culture Collection

**BSA** Bovine serum albumin

**CCD** Charge-coupled device

**FC** Flow cytometry

**FITC** Fluorescein isothiocyanate

**HMDS** Hexamethyldisilazane

**ICT** Information and communications technology

**IF** Immunofluorescence microscopy

**IgG** Immunoglobulin G

**InGaN** Indium gallium nitride

**IP** Intellectual property

**Iso** Isotype control

**LED** Light-emitting diode

**MF** Membrane filtration

**MLSB** Membrane lauryl sulphate broth

**MS** Mean squares

**NAP** National Access Programme, Tyndall National Institute

**NCLA** National Centre for Laser Applications, Galway

**PBS** Phosphate-buffered saline

**PCB** Printed circuit board

**PCM** Photon-counting module

**PDMS** Poly(dimethylsiloxane)

**PMMA** Poly(methylmethacrylate)

**POC** Point of care

**RS** Rabbit serum

**SEM** Scanning electron microscopy

**SD** Standard deviation

**Si** Silicon

**SS** Sum of squares

**SU8** Photoresist polymer

**TSB** Tryptic Soy Broth. Nutrient solution for cell growth

**UCC** University College Cork

**ZEPS** Department of Zoology, Ecology and Plant Sciences

**VCSEL** Vertical-cavity surface-emitting laser

# Appendix 1 List of Project Deliverables

As outlined in the End of Project Report, technical processing issues were encountered during the course of the work programme that prevented the demonstration and deployment of a fully functioning prototype cytometry system. This directly impacted upon the delivery of several deliverable reports which were contingent upon the demonstration of the final integrated unit. A more detailed explanation of these issues is provided in Section 3.3 of the report. All outstanding deliverables relating to the final system are highlighted in italics below.

**WP1 System Specification and Design**

***Tyndall National Institute***

D1.1 Specifications m3

D1.2 Initial designs m4

*D1.3 Final specifications and design files m36*

**WP2 Microsystem Fabrication**

***Tyndall National Institute***

D2.1 Initial microfluidics m9

D2.2 Initial optics m9

*D2.3 Updated parts throughout project m36*

**WP3 Microsystem Integration**

***Tyndall National Institute***

D3.1 Report on first-pass system assembly m12

D3.2 Report on basic control system m12

*D3.3 Final specifications and design files m36*

**WP4 Immunological Characterisation**

***ZEPS, UCC***

D4.1 Report on chosen systems and standards that will be trialled in the system m12

*D4.2 Report on performance of unit under differing conditions in light of modifications and updates m27*

**WP5 Biofouling and Cleaning Developments**

***ZEPS, UCC***

*D5.1 Report on effectiveness of options and improvements obtained m36*

**WP6 Project Management**

***Tyndall National Institute***

D6.1 Year one report m12

D6.2 Year two report m24

D6.3 Year three report m36

# Appendix 2 Presentations and Publications Pending

## Poster Presentations

Fogarty, B., Culloty, S., Davenport, J. and Alderman, J., 2005. *Development of a miniaturised portable microflow cytometry unit*. Nano-to-life Meeting, University of Twente, The Netherlands. 20–21 October 2005.

Fogarty, B., McCarthy, M., Culloty, S., Davenport, J. and Alderman, J., 2006. *Development of a miniaturised portable microflow cytometry unit*. Analytical Research Forum, Royal Society of Chemistry, University College Cork. 17–19 July 2006.

Fogarty, B., McCarthy, M., Culloty, S., Davenport, J. and Alderman, J., 2006. *Development of a low-cost miniaturised cell analysis system*. Nano-to-life Meeting, University College Cork, Ireland. 5–7 September 2006.

Fogarty, B., McCarthy, M., Culloty, S., Davenport, J. and Alderman, J., 2007. *Development of a multi-channel miniaturised portable microflow cytometry unit and its suitability for autonomous deployment*. Marine Institute, Oranmore, Galway. 31 March 2007.

Fogarty, B., McCarthy, M., Culloty, S., Davenport, J. and Alderman, J., 2007. *Microscale flow cytometry.* Nano-to-life Meeting, Lund University, Sweden. 22–24 October 2007.

Fogarty, B., Cranfield, C., Henry, W., Maaskant, P., Alderman, J., McCarthy, M., Duignan, L., Culloty, S., Davenport, J., Quinlan, F. and Jackson, C., 2008. *An integrated microfluidic flow cytometry unit for marine analysis*. ISAC (International Society for Analytical Cytology) XXIV International Congress, Budapest. 17–21 May 2008.

Fogarty, B. *et al.*, 2008. Alignment-free optofluidics for cellular analysis. Nano2Life Scientific Meeting. Heraklion, Crete. 25–27 June 2008.

McCarthy, M. and Culloty, S., 2006. *A comparison of a multi-channel flow cytometry system with existing techniques and its suitability for the detection of waterborne microorganisms*. Inaugural meeting of the Postgraduate Ecology Forum at University College Cork. January 2006.

McCarthy, M. and Culloty, S., 2007. *Screening for waterborne microorganisms: A comparison of a multi-channel flow cytometry system with existing techniques*. 2nd Annual Workshop on the Water Sensor Programme on 30 March 2007 at the Marine Institute HQ, Oranmore, Galway.

## Oral Presentations

Alderman, J., 2007. *Development of a multichannel miniaturised portable microflow cytometry unit and it's suitability for autonomous deployment*. Marine Institute, Oranmore, Galway. 31 March 2007.

Alderman, J. *et al.*, 2008. *Development of a miniaturised portable microflow cytometry unit*. Invited Oral Presentation. EPA Workshop. February 2008.

Fogarty, B. *et al.*, 2008. *Development of an integrated miniaturised flow cytometry unit*. Invited Oral Presentation, Lab Automation Conference, Palm Springs, USA. 27–30 January 2008.

Fogarty, B., 2008. *Microscale Flow Cytometry.* Invited seminar. University of Kansas. 1 February 2008.

## MSc Thesis

Moira McCarthy, 2008. *Optimisation of Immunological Techniques for the Detection of* Escherichia coli *in Suspension and their Application in the Development of a Miniaturised Flow Cytometry System*. Submitted June 2008 and passed July 2008.

## Publications in Preparation

MSc Thesis. Duignan, L., 2008. Title to be determined.

Duignan, L. *et al.* Paper on the microfouling performance of different construction materials exposed to different flow regimes at different depths. (In *Biofouling*, or in a materials journal.)

Duignan, L. *et al.* Paper on the influence of microtopography on microbial fouling. (*Biofouling.*)

Duignan, L. *et al.* Paper on microfouling succession upon glass slides in Lough Hyne at various sites, flow rates and depths.

Fogarty, B. *et al.* Invited review on microflow cytometry. Laboratory Medicine

Fogarty, B. *et al.* Invited review on miniaturised flow cytometry. Bioforum Europe.

McCarthy, M. and Culloty, S.C. Optimisation of two immunofluorescent antibodies for the detection of *Escherichia coli* using immunofluorescent microscopy and flow cytometry. *Current Microbiology*

McCarthy, M. and Culloty, S.C. Detection of water-borne *Escherichia coli* with flow cytometry and immunofluorescent microscopy, using two antibodies, and comparison with membrane filtration. *The Journal of Applied Microbiology.*

# 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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