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In Vitro Enabling Technologies for use in the Aquatic Environment

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A thesis submitted to the Dublin Institute of Technology in fulfilment of the requirements for the degree of Doctor of Philosophy.

Submitted by

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ABSTRACT.

As a result of an increasing emphasis on environmental protection, the growth of environmental awareness, an ever-increasing complexity of pollutants in the environment and the introduction of legislative measures, requirements for environmental testing and monitoring are increasingly prevalent. This is particularly true for the aquatic environment, where many pollutants end up. While testing programmes for this purpose appear to be very worthy causes, there is a huge reliance on *in vivo* based studies to qualify and/or quantify the state of the environment. These studies are typically based on the numbers of animals which survive various levels of exposures to tests and control substances. This practice is arguably, even less environmentally agreeable than the environmental questions which it is meant to address. Consequently, there is considerable focus on the development of *in vitro* based assay systems, particularly cell culture systems, to replace such practices. However, questions remain on whether these systems are truly representative of the environment they aim to assess.

The aim of this study was to develop alternative *in vitro* based systems which can replace the morally questionable and expensive *in vivo* testing practices while also addressing current *in vitro* based systems which tend to suffer from 'assay drift' and lack of relevance to the environment which it is monitoring. The study developed a number of tissue cultures for both vertebrate and invertebrate organisms and highlighted a middle ground whereby tissue cultures are used in a 'holding and treatment' system rather than establishing these cultures *in vitro* prior to treatment. While this system does not eliminate the use of live animals, significant reductions can be made depending on the tissue type investigated. Image analysis was adopted in clonogenic studies in this work to identify a bystander effect associated with rainbow trout tissue cultures. A number of molecular approaches were incorporated into the studies which focused on the activity and integrity of the mitochondrial genome following exposure to radiation, the model toxicant in the study.

This work highlights the application of gene expression using a convenient real-time PCR technique to identify alterations in the mitochondrial genome post-radiation treatment. This work provides several alternative approaches to reduce morally, economically and scientifically questionable live animal testing and which offers significant alternatives to comply with the ever-increasing raft of legislative measures introduced and emerging in recent and future years.

DECLARATION.

I certify that this thesis which I now submit for examination for the award of Ph. D, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work. The use of immunocytochemistry in the characterisation of cell lines section in Chapter 2 was performed by Dr. Peter Olwell.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, lend or copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature Colm O'Dowd Date 12/9/11

Candidate Colm O'Dowd

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Finally, a big thanks to my family especially my wife Leonie, the kids, Ruan, Veronica, and Lir, my mother, Rita and my brother Brendan for their sustained support and encouragement throughout this episode of my life.

DEDICATION

This thesis is dedicated to my mother Rita, who is currently in hospital but should be well enough in time to attend the graduation party!

LIST OF ABREVIATIONS.

RPMI	Roswell Park Medical Institute
DMEM	Dulbeccos Modified Eagles Medium
L-15	Leibovitz
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
CHSE	Chinook Salmon Embryo
EPC	*Epithelioma papullosum cyprini*
BF-2	Blue gill sunfish
RTG-2	Rainbow Trout Gonad
MEM	Modified Eagles Medium
FBS	Foetal Bovine serum
^{40}K	Potassium-40
Bq	Becquerel
Gy	Gray
J	Joule
^{60}Co	Cobalt-60
mtDNA	Mitochondrial DNA
FDA	U.S. Food and Drug Administration
IMM	Inner Mitochondrial Membrane
ROS	Reactive Oxygen Species
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ETC	Electron Transport Chain
H^+	Hydrogen ion

P*i*	Inorganic Phosphate
kbp	kilo base pairs
bp	base pair
CD	Common Deletion
tRNA	transcription Ribonucleic Acid
PCR	Polymerse Chain Reaction
HSP 60	Heat Shock Protein 60
RESC	Radiation and Environmental Science Centre
SSW	Sterile Seawater
PBS	Phosphate Buffered Saline
OD	Optical Density
OXPHOS	Oxidative Phosphorylation
CAMK	Calcium-calmodulin kinase
mtTFA	Mitochondrial Transcription Factor A
AMPK	5′-AMP activated protein kinase
RFLP	Restriction Fragment Length Polymorphism
ddH_2O	Distilled Deionised Water
DTNB	5'5'-dithio-bis-(2-nitrobenzoic) acid
CS	Citrate Synthase
SSCP	Single Strand Conformational Polymorphism
MMP	Mitochondrial Membrane Potential
ITCM	Irradiated tissue conditioned medium
ICCM	irradiated cell conditioned medium
IR	Ionising Radiation
TCA	Tricarboxylic Acid

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CHAPTER 1. GENERAL INTRODUCTION

1.1 General Background

There is growing interest in the protection of the environment not only for the purposes of maintaining the quality of life but also from the realisation that there are costly economic impacts associated with its degradation. Given that more than 70% of the earth's surface is under water, the importance of this resource has received significant attention in recent years (1). Aquatic resources are a necessary component of life and are exploited recreationally and commercially in a plethora of ways. Improvements in information relating to pollution in the aquatic environment have generated an informed and exacting society, focussed on the protection and ecological relevance of this environment.

Consequently, this has highlighted the need for the regulation and protection of the aquatic environment qualitatively and quantitatively going forward. However, the aquatic environment is highly complex with inputs from large areas, diverse habitats, industrial conurbations and populous centres. Coupled with these spatial and socio-economic aspects, the physical nature of the local environment further adds to the complexity due to the temporal and physicochemical interplay between the aquatic resource and its environment.

In an effort to provide for this complexity within a test system, most studies relating to both natural and anthropogenic pollutants in the aquatic environment incorporate various organisms from different genera, families and phyla associated with that environment. Organisms typically include members of the micro and macroalgae, bacteria, protozoa, invertebrate and vertebrate families (2-4). This 'battery' of test species aims to mimic the trophic structure existing in the ecosystem being studied.

This may justifiably provide a realistic and accurate evaluation of the impact of a pollutant within an ecosystem. Given that the test substances / pollutants may be chemical, biological or physical in nature (1) and present in combination, in exposures ranging from chronic to acute, and that it is necessary to examine a particular ecosystem thoroughly, thousands of such tests are carried out routinely each year. While this approach is a good indicator of lethal dose concentrations of toxicants in a water body, significant shortcomings persist. Many millions of fish are sacrificed annually in laboratory-based experiments. This practice is morally and ethically questionable, economically costly and indeed may be difficult to validate scientifically.

Increasingly, many studies focus on the development of *in vitro* based assay approaches which attempt to provide for the diversity and complexity of the aquatic environment without the sacrifice of animals. These assays frequently involve the use of *in vitro* based cell cultures associated with representatives from the trophic structures of conventional toxicity tests described above (5-7).

Several *in vitro* based cultures are already in use (8-9) however, as aquatic ecosystems are increasingly endangered, a greater diversity and application of cell and tissue culture is required. Results obtained must be indicative of these particular ecosystems and their state of pollution. There are numerous and ever growing applications of cell and tissue culture. Previously, cell culture was pre-requisite for the growth and maintenance of viruses and some bacterial pathogens (10). Currently, applications include pathology diagnosis, efficacy studies, environmental monitoring, genetic studies, bioactive compound production, biomimetic production and cellular and molecular function and response studies (11-14).

1.2 Cell and Tissue Culture of Aquatic Organisms

The development of cell culture may be traced back to 1907 when Harrison reported growing frog nerve cells using the hanging drop technique (15). Since then, vast arrays of taxa have been used to develop cell lines and tissue cultures. Continuous cell culture requires the demonstration of reproductive division of immortal cells from a single cell origin.

Primary tissue culture is defined as the outgrowth of relevant cells from tissue fragments or suspensions (16, 17). Primary tissue culture involves plating small pieces of tissue in culture media, which have been excised directly from the animal. Once attached to the flask, cells will migrate from this tissue and spread outwards from the explant over a number of days or even months. Such cells never propagate themselves. The benefit of using primary explant cultures is that the multi-cell type nature of the organ is retained. Conversely, continuous cell cultures are cells, which have 'evolved' from primary culture. They may be transferred to new flasks and are able to propagate and sustain themselves independently, given suitable conditions. The term 'evolved' must be used carefully. Unlike primary cell cultures, cell lines often lose many of the traits, which are associated with the original tissue from which they are derived and may include functional structural and metabolic processes (18).

Little is known about the nutritional requirements of tissue and cell culture of aquatic species. Often, commercial mammalian growth media is added to fresh or salt water as a starting point (17). Typically, Roswell Park Medical Institute (RPMI) Medium, Dulbeccos Modified Eagles Medium (DMEM) or Modified Eagles Medium (MEM) are used as a basis for vertebrate media while Leibovitz (L-15) media is the preferred choice for the culture of invertebrate cultures (16). Serum, amino acids, vitamins and various growth factors may be added to this in varying quantities on a trial and error

basis to optimise growth of any particular tissue (17). It is vital however, that the pH and osmolarity of the media are adjusted to appropriate levels. This is often determined by equilibrating to those levels found in the haemolymph (19).

1.2.1 Applications of tissue/cell cultures in aquatic toxicology

Many studies to date have relied on the establishment of tissue and cell cultures prior to their inclusion in toxicology studies. The effect of a test substance is then assessed by the extent of proliferation or inhibition in the outgrowth or confluency of these indicator cultures. *Ex vivo* tissue cultures are generally considered to be 'established' when there is cellular outgrowth from the explant. Cell cultures are established when there is an even growth of cells over the surface of the flask. Once tissues or cells are established *in vitro*, the system may be used to test a broad range of hypotheses and in many different applications. In toxicology studies specifically, these cultures allow for a uniform dose exposure to each cell, facilitating accuracy and repetition in experimental analyses. Recognition of this has increased the importance of cell culture over the past number of years. The literature reports many different uses for *in vitro* cell cultures. Primarily, uses include screening or toxicity ranking for various substances, development of structure-activity relationships and replacement of *in vivo* animal testing (7, 20). Others include the maintenance and study of viruses and bacterial pathogens, cell function, cell–cell signalling and interaction, and the production of pharmaceutical or bioactive compounds (7, 16, 21, 22).

Due to increasing awareness of the environment and the on-going introduction of ever more-stringent environmental legislative measures, methods for assessing the aquatic environment are increasingly important and require innovative approaches to meet the diverse challenges which pollutants now present. Scientists are challenged to meet the needs of those governing authorities, who not only require systems which monitor

complex substances but also systems with accurate endpoints, representative of the environmental conditions which persist. The toxic effects of chemicals on *in vitro* cell cultures may be assessed using apoptosis, necrosis and expression of proteins associated with genotoxic damage (5, 23, 24). Other endpoints used to monitor toxic effects include cell morphology, cell attachment, total protein levels, gene regulation studies and clonogenics (7).

Underlying the development of cell and tissue culture in eco-toxicological applications are growth, characterisation, validation and utilisation. Once specific factors in each of these areas are met, a technology for aquatic toxicology can be developed.

The use of sections of organ excised from the animal and cultured in flasks is a questionable approach given that each organ may comprise of several tissue and cell types. However, in short term experiments, such practice is particularly useful given that these sections remain intact, are associated with a specific function within the organism and any increased outgrowth/propagation is easily discernable. Furthermore, the sections generally comprise enough cells to yield adequate amounts of DNA/RNA for standard genetic analyses, unlike many primary cell cultures.

1.2.3 Choice of Tissues

The initial choice of species is an important factor to consider when developing eco-toxicological assays using tissue cultures. A logical approach to this is to identify organisms which fulfil the role of indicator species and live in the environment which requires monitoring.

It may be safe to assume that cells from an organism that can survive in polluted waters are ‘conditioned’ and are consequently more likely to withstand similar contaminants *in vitro*. If this is correct, then choosing a species to develop an eco-

toxicological assay is less complicated. The literature suggests a number of traits, which the researcher should consider prior to adapting a species for *in vitro* eco-toxicological use. A species indigenous to a wide geographic region means that a system, once developed, may be used over greater areas, generating more meaningful and comparative data (25). Equally, if the organisms are susceptible to both lethal and sub-lethal quantities of a wide range of contaminants *in vivo*, then comparative studies may be carried out with tissue cultures from these animals. It is important that tissues are selected that can withstand measurable quantities of the contaminants in question (26). The chosen species should be easy to collect so that a ready supply of tissue is available (27). Tissue isolated from a variety of faunal classes and feeding types may optimise the ability of that tissue to withstand a wider variety and a higher concentration of contaminants associated with polluted environments. In addition to this, tissue from a species which is commercially important, adds value to the significance of the research (28). In the aquatic environment, candidates from vertebrate and invertebrate groups should be chosen (29). Finally, the organisms must be easy to maintain in the laboratory without compromising the condition of the organism (30).

1.2.4 Vertebrates and Invertebrates

One of the main limiting factors in developing technologies in aquatic toxicology is the dearth of tissue cultures available. While an ever-increasing number of aquatic vertebrate tissue cultures are developed, there are few freshwater invertebrate cultures and no marine invertebrate cell cultures (31, 32). Several authors report success with marine invertebrate cell cultures however, these all are short lived. A typical example being the cell line derived from the sea squirt, *Ciona intestinalis* (33). The sustainable culture of marine invertebrates remains elusive to date. Compounding these factors

further is the fact that much of the pollution, which requires monitoring, is adsorbed to and 'locked' in the sediments, often the very environment which invertebrates inhabit.

1.2.5 Vertebrate Cell/Tissue Cultures

Cultured, immortalised fish cell lines, which include Chinook Salmon Embryo (CHSE) cells, *Epithelioma papullosum cyprini* (EPC) cells, Blue gill sunfish (BF-2) cells and Rainbow Trout Gonad (RTG-2) cells, have been used for many years (5, 34 – 37). Rainbow trout (*Oncorhynchus mykiss,* Walbaum) is chosen as a general investigative model of aquatic species by many researchers. Rainbow trout tend to be easy to work with and are easily maintained in laboratory conditions. Organ tissues from the Rainbow trout, which have been cultured to date, include skin, gill, spleen, gonad, kidney, fin, and gut amongst others. Generally researchers have found varying success depending on the choice of tissues, which are cultured. However, the literature reveals much success with rainbow trout species overall (1, 38 – 41).

1.2.6 Invertebrate Cell/Tissue Cultures

The blue mussel (*Mytilus edulis)* has been used for over 30 years to monitor environmental health conditions by means of the 'Mussel Watch' programme (42). Their use in toxicology and eco-toxicology has developed rapidly. They are beneficial due to the large volume of water they filter and their wide geographical distribution (28). Certainly, the development of a mussel cell line would be invaluable in furthering the field of eco-toxicology due to the comparative data, which is available. Some developments have been made in tissue culture of marine invertebrates cultured cells from the larvae of *M. edulis galloprovincialis* however these cultures survived

for just 2 weeks (43). Le Marrec *et al.* (1995), cultured cells from the heart and gills of the scallop, *Pecten maximus* (44).

Mulford and Austin (1998), developed primary cultures from the ovary, testes, hepatopancreas, haematopoietic tissue, heart, gut, gill, eye-stalk and nerve tissues of the Dublin Bay prawn (*Nephrops norvegicus)* (45). A number of different media including Leibovitz medium (L-15), Modified Eagles Medium (MEM) and Medium 199 (M199) were used with varying levels of supplements. The best results were achieved with ovarian tissues, which were maintained in 2x L-15 with 5% foetal bovine serum (FBS). One subculture was achieved prior to the collapse of the culture (45). Mothersill *et al*. (2000) outlines that success with cell cultures is closely correlated to the use of young actively growing animals (19). Indeed, Hansen (1976) developed the first invertebrate cell line from the larvae of *Biomphalaria glabrata* (46). There are many instances of embryonic and larval stages of the life cycle used to generate viable tissue cultures (47, 48, 43, 44). Whatever the reason, all of these cultures were relatively short-lived. Developing a cell line from a marine invertebrate has remained quite elusive to date. This has restricted the development of *in vitro* based toxicology testing, as much of the preliminary work when developing a test system focuses on the development of a reliable culture system rather than investigating the effects of toxicants on that system.

In the interests of investigating alternative *in vitro* tissue based toxicity test systems, γ radiation as a model toxicant system was developed. The rationale for using radiation is clear. The level of dose is conveyed to all cells uniformly in a reproducible manner throughout the tissue explant. This is not achievable with a chemical toxicant especially using explants. Additionally, this is a safe experiment to perform and reduces the need to handle dangerous chemicals at high concentrations.

1.3 General Radiobiology

Radioactive pollution in the aquatic environment is introduced through both natural and anthropogenic channels. Background radiation predominantly arises from the presence of potassium-40 (^{40}K) in seawater however there are several other radionuclide representatives found naturally in seawater including tritium (^{3}H), Rubidium-87 (^{87}Rb, Uranium (^{234}U and ^{238}U) and Polonium-210 (^{210}Po) (1). Anthropogenic sources include cooling waters discharged from nuclear power plants, reprocessing plants, nuclear powered vessels, and leakage from ocean dumping programmes [1].

1.3.1 Principles of Radioactivity

Many atoms have isotope forms. These isotopes have the same number of protons in the nucleus but the number of neutrons may vary. For example, the stable form of potassium (Potassium-39) contains 19 protons and 20 neutrons in the nucleus. However, its isotope, Potassium 40 (^{40}K), is unstable having 21 neutrons. Typically, this instability is rectified, within unstable isotopes, with a spontaneous change in the ratio of protons to neutrons. This process is associated with an emission of particles or energy known as radioactivity (1). The unstable isotope forms of atoms are known as radioisotopes or radionuclides. Each radioisotope has its characteristic form of emission. Alpha (α) radioactivity is characterised by slow moving particles and is easily stopped by paper for example. Beta (β) particles occur when a proton spontaneously changes into a neutron or *vice versa*. These particles are slightly more penetrating but may be contained using light perspex. Conversely, gamma (γ) radiation is deeply penetrating and requires lead or concrete to contain it. While α and

[1] Dumping radioactive waste at sea continued from 1946 until 1983 when a ban was introduced following the London Dumping Convention (Laws, 2000).

β particles may be easily contained, they are nevertheless, intensely ionising if they enter living tissues. Ingestion or inhalation can cause severe damage. α particles, if ingested, are estimated to be, on average, 20 times more damaging to living tissue than β or γ radiation. While γ radiation may be considered less severe, it has the added danger of freely penetrating most tissues (1). The International System of Units (SI) for measuring radioactivity is the Becquerel (Bq). Becquerel measures the rate at which radioactive disintegrations of the nuclei of an isotope take place. One Bq is equivalent to one dis-integration per second. Therefore, radioactivity of a substance reduces over time. The half-life, or the time it takes for the radioactivity of a substance to be halved, may be calculated for each isotope. In biological studies however, it is also necessary to have a measure of the amount of radioactivity, which a tissue absorbs. The Gray (Gy) is defined as the amount of radiation required for 1 kilogram (Kg) of tissue to absorb 1 joule (J) of energy.

1.3.2 Radioactivity in the Aquatic Environment

The Earth's environment is naturally radioactive with cosmic radiation emitted from space and terrestrial radiation emitted from the earth's crust. While ^{40}K, may be responsible for the vast proportion of background radiation in the marine environment, radiation levels in the aquatic environment often exceed these levels due to emissions from a variety of anthropogenic sources including power stations, reprocessing plants, nuclear powered vessels and hospitals (49). Many radioisotopes, particularly those with high atomic mass tend to adsorb onto marine sediments. Cobalt-60 (^{60}Co) is one such isotope. ^{60}Co is a γ-emitting radioisotope. It is of particular interest in the marine environment due to its relatively long half-life (5.3 years), its high-energy γ emissions and its tendency to adsorb to sediments in the

marine environment. It is monitored in many harbours and dockyards in Britain where nuclear vessels are stored and maintained (4).

The predominant effect of radiation on living organisms ultimately manifests itself in DNA damage. Mutations and deletions in the structure of DNA can be lethal or may result in sub-lethal deformity or dysfunction in the organism. The mitochondrial genome is particularly susceptible to DNA damaging agents due to the absence of a protective histone coat, its decreased repair capacity and the lack of recombination characteristic of mtDNA (50).

1.3.3 The Bystander Effect

The 'bystander effect' is a phenomenon where cells never exposed to radiation display radiation like damage if in the vicinity of an irradiated cell or exposed to growth medium from irradiated cells (51). The schematic in Figure 1 represents the effects of direct radiation and indirect radiation on the integrity of the exposed cells and their progeny. Direct radiation, in this case, is by exposure to γ radiation while indirect effects are the result of exposure of the cells to Irradiated Cell Conditioned Medium (ICCM). The process involved in conferring this effect is not yet known. However, the transfer of the cytoxic activity within the media used during cell irradiation suggest that it may be a signalling factor released by the cells (52). Whatever the mechanism, it has implications for aquatic species exposed to ionising radiation. Similarly, the bystander effect has implications for aquatic food products that are irradiated to remove undesirable microorganisms or to prolong shelf-life. Recently, the U.S. Food and Drug Administration (FDA) approved the use of ^{60}Co and electron beam γ radiation treatment, to remove *Vibrio vulnificus* from cultured oysters prior to sale (53, 54).

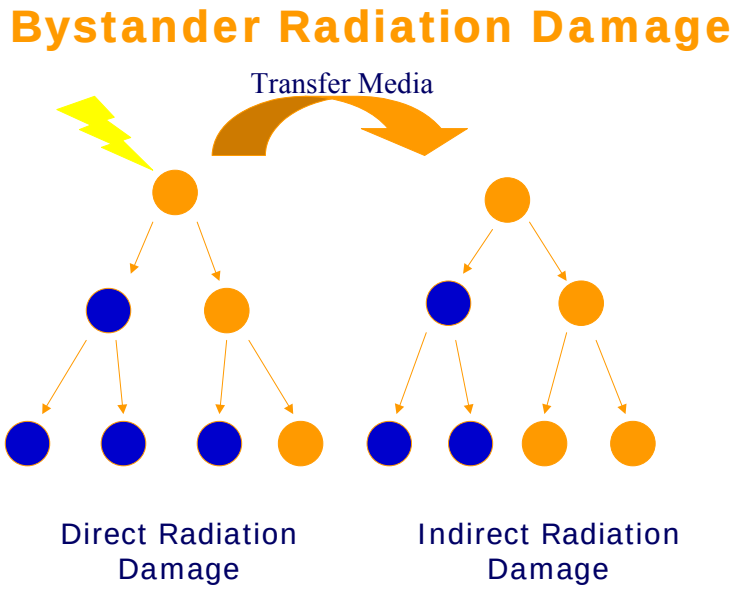

Figure 1.1 Schematic representations of the effects on cells of direct radiation and indirect radiation via exposure to media used to culture irradiated cells

1.4 The Mitochondrion.

The mitochondrion has received renewed interest from molecular and cell biologists in recent years due to an ever increasing battery of techniques based on mitochondria which have been developed in this period. Not only is this due to its importance in energy production in the cell but increasingly due to its association with apoptosis and the production of reactive oxygen species (ROS) among other functions. Mitochondria are quite sensitive to damage however, while cells containing mitochondria with substantially damage mtDNA can often operate without loss of overall cell function.

The mitochondrion is found in cells of all eukaryotic species including plants and animals. It is almost entirely maternally inherited being passed in the cytoplasm of the oocytes during fertilisation. Several copies are transferred via the sperm however are lost during fertilisation or are diluted out shortly afterwards during replication (55, 56). It is widely accepted now that mitochondria entered the proto-eukaryotic lineage in a symbiotic relationship more than 7 billion years ago during the onset of transition

towards an aerobic environment. There is also some evidence that mitochondria descended from the family of purple bacteria (57, 58).

1.4.1 Structure of the mitochondrion.

Structurally, the mitochondrion may be polymorphic but generally is ‘capsule like’ in shape. The shape depends on the tissue or cell types in which it resides, and in particular is dictated by the degree of energy requirement on that tissue at a given moment. It is enclosed by a double membrane. The outer membrane is smooth and contains many porin proteins which confer permeability to many molecules in the cell cytoplasm, allowing them to move freely across the outer membrane. Conversely, the inner mitochondrial membrane (IMM) is selectively permeable and can restrict the movement of protons and other ions across it. The sector contained within the inner mitochondrial membrane is termed the matrix and contains a host of enzymes, copies of the mitochondrial genome, and the machinery responsible for the transcription and translation of the mitochondrial genes. This plays an important role in the functioning on the oxidative phosphorylation (OXPHOS) process which is embedded in the IMM. The IMM is folded into finger like projections called cristae, thus greatly increasing the surface area of the inner membrane. An increased level of folding facilitates greater energy production and consequently is a determining factor in the shape of the entire structure.

1.4.2 Function of the mitochondrion

The mitochondrion is generally associated with energy production although, it is more recently known to facilitate other processes in the cell including apoptosis through the production of pro and anti-apoptotic factors (Bcl-2, AIF, pro-caspase) and ROS production and cytochrome c release.

Energy is stored in cells by means of high-energy phosphate bonds, formed in molecules of adenosine tri phosphate (ATP). ATP is synthesised from adenosine diphosphate (ADP) by the addition of a phosphate group. This can occur in the cell cytoplasm in a process known as glycolysis or in the IMM of the mitochondria as the final product of oxidative phosphorylation.

Glycolysis is an anaerobic process where glucose is broken down in a series of reactions to form pyruvate. Each reaction produces a hydrogen ion, which is used to make an ATP molecule. Each molecule of glucose realises 4 molecules of ATP following the glycolytic pathway.

Conversely, the aerobic process of OXPHOS carried out in the mitochondria realises up to thirty molecules of ATP from one molecule of glucose, representing a much more efficient conversion of the chemical energy harnessed within food stuffs. This is the reason why the mitochondrion is sometimes referred to as the 'powerhouse of the cell'. In preparation for OXPHOS, pyruvate produced by glycolysis, and fatty acids, stored as triglycerides, are selectively transported from the cytosol into the mitochondrial matrix. Pyruvates and triglycerides are converted to an acetyl group by pyruvate dehydrogenase complex and the β oxidation pathway respectively. This acetyl group enters a series of complex reactions known as the Citric Acid Cycle (also known as Krebs Cycle). The 2-carbon acetyl group combines with a 4-carbon oxaloacetate molecule to form a 6-carbon citrate group. The reactions within this cycle remove four pairs of electrons; three of which are transferred to NAD^+ to form three NADH and one to FAD to form $FADH_2$.

NADH and FADH ferry the electrons to the electron transport chain (ETC). The ETC is a series of enzyme complexes (Complex I – IV), which are located in the IMM as depicted in Figure 1.2. A proton pump is located at each of Complexes I, III and IV,

where protons (H^+) are pumped across the IMM, creating a proton gradient across the membrane. The ETC) along with ATP Synthase (Complex V) constitutes the OXPHOS pathway.

Complex I and II receive electrons (e^-) from NADH and FADH respectively. Ubiquinone transfers electrons from Complexes I and II to Complex III. Cytochrome c transfers electrons from Complex III to Complex IV where they are captured by oxygen to form water (½ O_2 + e^- + H^+ => H_20

Thus the chemical energy of glucose is converted by the mitochondria to potential energy in the form of a potential difference (typically -34mV across the IMM). The dissipation of this proton gradient across the IMM through ATP synthase is coupled to the fusion of ADP and inorganic phosphate (P*i*) forming the energy rich ATP molecules (59 - 62) and thus the potential energy of the proton gradient is converted back into chemical energy in a form more readily usable by energy demanding processes of the cell.

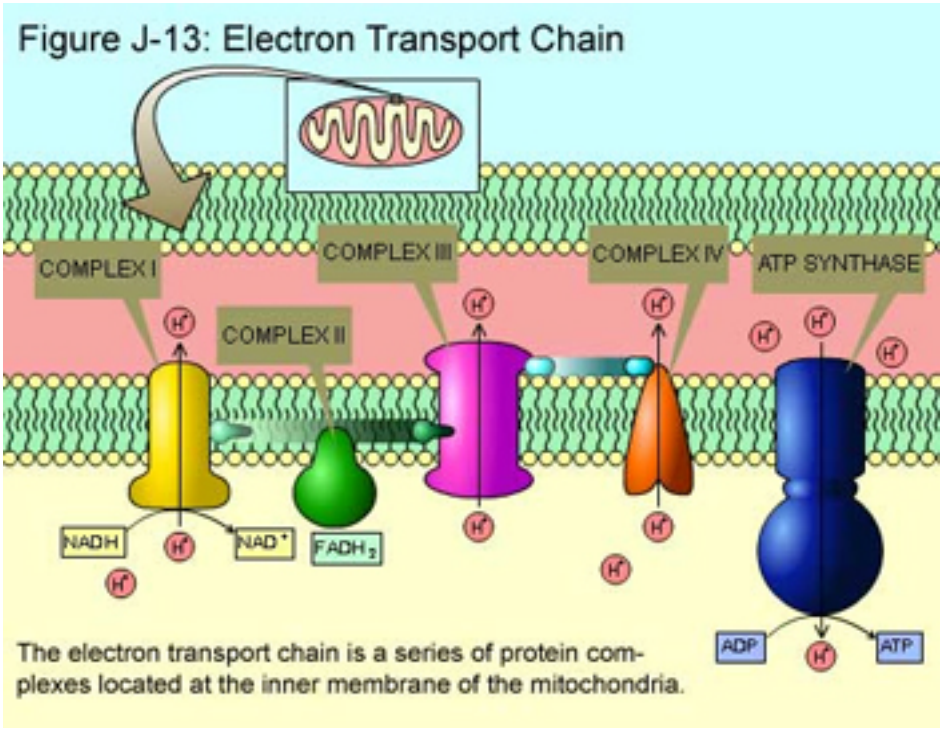

Figure 1.2 Diagram depicting the Electron Transport Chain and the process of oxidative phosphorylation.

(taken from http://www.progressivegardens.com/knowledge_tree/electrontransportchain.jpg)

1.4.3 Genetics of the mitochondrion.

The mitochondrion has its own genome and is able to replicate, transcribe and translate its own DNA independent of nuclear DNA (nDNA). Indeed, the mitochondrion is the only other location where DNA exists in the cell outside of the nucleus. However, mitochondrial and cellular functions are interdependent. The mitochondrial genome encodes thirteen of the eighty-seven proteins necessary for the oxidative phosphorylation process, though the enzymatic machinery required must be imported into the mitochondrion from the cytosol. The sizes of the genomes differ depending on the species. To date, sequencing data shows that smaller genomes are generally associated with higher organisms although this is somewhat of an oversimplification. For example the smallest genome sequenced to date has been that of the Mosquito, *M. falciparum* at 5.6 kilo base pairs (kbp). The human mitochondrial genome is 16,569 base pairs (bp) and the genomes of some plants are in the region of 2000 kbp.

The mitochondrial genome is highly conserved intra species. An example of this is the considerable consensus, which exists between the human mitochondrial genome and the Rainbow trout mitochondrial genome. The rainbow trout genome is 16,642bp in length and like all mtDNA, is arranged in a circular, double stranded molecule containing thirty-seven genes. Thirteen genes code for structural proteins of OXPHOS while the remaining twenty-four genes encode for RNAs associated with the translation of the mtDNA structural genes. Unlike nDNA, mtDNA has no introns and is almost entirely made up of coding regions. Other disparities exist between nDNA and mtDNA. A normal cell only contains one copy of nDNA. One mitochondrion may contain between two and ten copies of the genome while one cell may contain from several to one hundred mitochondria (60). This provides for the

existence of up to several thousand copies of the mitochondrial genome in one cell (63).

MtDNA is more prokaryotic in nature than eukaryotic. It is more susceptible to mutations for a number of reasons. It employs prokaryote-like codons and has no protective histone coat. Given that the mtDNA is almost entirely made up of these coding regions, including some overlapping genes, point mutations or deletions typically give rise to phenotypic effects. Moreover, the genome is located in close proximity to a significant potential source of free radicals, as electrons are passed from carrier to carrier on the ETC, which is embedded in the IMM. MtDNA also possess limited proofreading abilities. Murphy *et al.* (2005) suggest that these factors are responsible for the anomaly between the mitochondrial genome and the nuclear genomes: a ten- to twenty-fold greater mutation rate (64). While the mitochondrial genome encompasses a minute fraction of the total genetic material in a cell, any damage or alteration to it can still have serious implications for a cell's viability and/or survival.

However, while damage may occur in several copies of the mtDNA, the existence of a high total copy number means that not all of the mtDNA genomes in the cell are necessarily affected. This means that even within one cell, both normal (wild type) and mutant mtDNA are present and indeed are typically of a multi-variant nature. In humans, this characteristic is known as heteroplasmy. A cell may survive with considerable mutant mtDNA however there is a ‘threshold level’ and once the frequency of a mutant mtDNA surpasses this, oxidative phosphorylation may be compromised and a phenotypic disorder results (60) although this threshold level is very high and for example as much as 70% of the activity of Complex I can be lost

before OXPHOS activity is affected and the performance of the cell compromised (65)

Mutations and deletions in mtDNA are common. The diagram of the human mitochondrial genome in Figure 1.3 also depicts the common deletion (CD), a deletion feature that has derived its name from the frequency of its occurrence (66, 67). The common deletion has only been studied in human and mouse mtDNA to date and results in a 4977 bp sequence being lost from the mitochondrial genome. Several genes, essential to the function of the mitochondria are lost during this phenomenon. These include four polypeptides for complex I (ND3, ND4, ND4 L & ND5), one for complex IV (COIII) and two for complex V (ATP 8 & ATP 6) and five tRNA genes (68)

In recent years, there has been growing interest in the mitochondria and its (dys)function (60, 62, 69). It is becoming apparent, that they may be utilised in providing a platform for biomarkers to indicate toxic stress. Sweet *et al*. (1998) amongst others, has identified several such potential targets for toxicity in the cell (70, 71). These include the susceptibility of the mtDNA to mutagens and the association of mitochondria with proteins critical to stress and death responses. Nugent *et al.* (2007) has reported an increase in mitochondrial mass and mitochondrial genome number in cells exposed to radiation (72). Typically, hundreds or even thousands of copies of mitochondrial genomes can exist in single cells.

Deletions such as the common deletion are detrimental to the mitochondria. Heteroplasmy allows the mitochondria to function until a threshold level of mutation is reached. Hayashi *et al*. (1991) demonstrated that the threshold level for mutant DNA is 60% and this is sufficient to inhibit overall mitochondrial function (73). However, many cells carry lower levels of the CD without any loss of function. Cells

in tissues/organs which, because of their functional requirements, demand greater energy levels (e.g. gill in fish) are more likely to have lower threshold levels than cell which do not require high levels of energy (e.g. spleen). PCR (Polymerase Chain Reaction) may be used to calculate the ratio of mutant to wild type genomes present in the organelle. Using this technique, very low frequencies (as low as 1 in 10,000) of the CD can be detected.

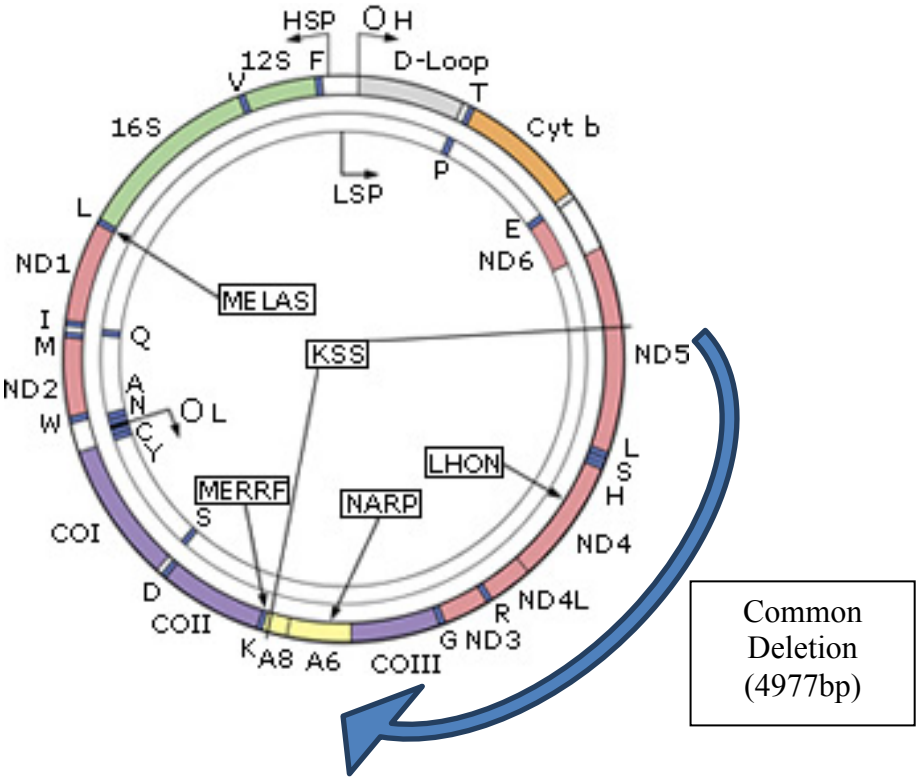

Figure 1.3 Schematic representation of the position of the common deletion found on the human mitochondrial genome (mitochondrial genome structure

(taken from www.columbiamitodiagnostics.org/overview.html)

Several stress response proteins and apoptotic protein markers are associated with the mitochondrion. In the case of stress response, heat shock proteins, particularly HSP 60, stabilize unfolded or partially folded protein structures (74). Member of the Heat Shock Protein family including HSP 60, are over-expressed in fish in the presence of stressors such as heat and confinement (75). Identifying increases in levels of expression of this protein can serve as an indicator of toxicity (71).

The anti-apoptotic Bcl-2 protein is mainly located on the outer mitochondrial membrane surface. Cytochrome-c, a highly conserved protein, is typically present in the inter-membrane space (76). Their functions are associated with apoptosis (programmed cell death). Apoptosis, which is induced by certain agents, may be prevented by an overproduction of Bcl-2 within the cell. Conversely, Cytochrome-c is required for activation of apoptotic caspases (77). Prior to apoptotic cells appearing, cytochrome-c levels increase in the cytosol. Sweet *et al*. (1998) suggest that monitoring Bcl-2 and Cytochrome-c in the mitochondria may be useful markers in the early identification of a toxic response in the cell (70).

1.4.4 Mitochondrial dysfunction

The process of OXPHOS occurs within the mitochondria of cells and employs a series of enzyme complexes (complexes I-V) embedded in the IMM. This series of protein complexes carries electrons along the inter-membrane space while pumping protons across the membrane to create a proton gradient within the organelle. The kinetic efficiency of each of these complexes may be measured. An imbalance or lack of activity within these complexes may correlate to increases in toxic stress causing mitochondrial dysfunction.

In some human mitochondrial myopathies, mitochondrial dysfunction manifests itself with a proliferation of the organelle (66). Identifying this quantitatively or even

qualitatively can therefore identify toxic stress (78). Advances in semi-quantitative PCR or real-time PCR techniques may have useful applications to this end.

Similarly, proteomics is a useful tool in the development of assay systems for assessment of aquatic toxicology. Proteomics gives a quantitative picture of the protein expression and changes therein following toxic assault (79, 80) identified an increase in mitochondrial gene expression following oxidative stress. Up-regulation and down-regulation of gene expression are the cornerstones of toxicogenomics. Toxicogenomic studies have recently become popular as a method to detect toxic stress (81). Changes in gene expression following toxicant exposure are readily identifiable markers (80). Furthermore, recent developments of DNA microarrays have allowed the possibility of investigating the reaction of hundreds or even thousands of genes against a wide range of toxicants (80). In an effort to fully understand the potential of the mitochondria in toxicity studies a closer look at the structure, function and genetics of this organelle is required.

1.5 Aims of the thesis.

The aim of this thesis was to further develop *in vitro* assay systems for application in aquatic eco-toxicology monitoring and control. Currently, live whole animal studies are used in the assessment of pollution and toxicant exposure in the aquatic environment. An *in vitro* assay system would significantly reduce the number of animals required for the generation of similar levels of data. Moreover, the development of *in vitro* assays may be an effective alternative, in terms of cost, time and ethics when compared to expensive, time consuming and morally questionable live animal experiments.

The literature review identifies the mitochondria and their associated cellular characteristics, functions and processes as an organelle with increasing significance in toxic exposure studies. It offers several features which couple as putative biomarkers, including a unique genome, an electron transport chain and two membranes containing several proteins of importance in cell defence mechanisms. Test model systems investigated here included an explant culture system and a focus on the damage and dysfunction of the mitochondrion. This system can be applied across a range of species and trophic levels in environmental monitoring studies.

In terms of aquatic toxicology, the literature review identifies an obvious gap in the understanding of radioactivity and radioactive pollution in the aquatic environment. While current environmental programmes identify safety levels for humans in the aquatic environment, little or no work has been carried out on the effects of radioactive pollution on aquatic organisms or the cellular responses of organisms exposed to radioactivity. The Radiation and Environmental Science Centre (RESC) has significant experience in Radiation Biology and the effects of radiation in the environment.

In light of these permutations, this research study aimed to apply *in vitro* tissue culture systems, based on aquatic species, to the development of novel mitochondrial biomarkers using γ radiation as a test model.

Specifically;

- To develop further tissue cultures, which are sustainable, reliable, appropriate and manageable and apply these cultures in aquatic eco-toxicology, through the development of novel assay systems.
- To examine various tissue types including skin, gill and spleen from vertebrate species and gonad, pallial mantle and gill from invertebrate species for tissue culture systems.

- To investigate the potential of putative *in vitro* based technologies as biomarkers using γ radiation as a test model of toxic stress
- To investigate *in vitro* applications using fish tissue/cell cultures to examine the suitability of these cultures as a model system to assess bystander factors
- To investigate the suitability of tissue culture techniques in the study of mitochondrial (dys)function in tissues exposed to radiation *in vitro*.
- To investigate the application of mitochondrial gene regulation in the area of Radiobiology

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CHAPTER 2 THE DEVELOPMENT OF *IN VITRO* ENABLING TECHNOLOGIES FOR USE IN AQUATIC TOXICOLOGY.

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4 Figures, 3Tables

In vitro enabling technologies for use in aquatic toxicology.

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2.1 Abstract

O'Dowd, C., Olwell, P., Mothersill, C.E., Cairns, M.T., Austin, B., Lyng, F.M. and Murphy, J.E.J. The development of *in vitro* enabling technologies for use in aquatic toxicology.

There is a growing interest in the use of *in vitro* cultures of aquatic vertebrates and invertebrates for use in monitoring the condition of the aquatic environment. This is motivated not only by ethical and moral concerns but also by increasing economic pressures associated with live animal toxicity testing.

While there have been significant developments in tissue and cell cultures over the past number of years, shortcomings still exist. These mainly centre on the reliability and robustness of tissue cultures, the identification and contamination associated with initiating cell cultures and the lack of cultures available, in particular, aquatic invertebrates and those from the marine environment.

Here we report on a number of techniques which aim to progress the application of cell and tissue culture in eco-toxicology and discuss their merits in that context. In particular, we examine the process of culturing cells from fish and shellfish organs and larvae and review the use of *in vitro* tissue culture as a holding and treatment facility rather than a culture platform. RNA and DNA extracted from γ irradiated cultures were assessed qualitatively and quantitatively for further downstream applications in ecotoxicology as a model system. We also show applications of molecular biology and immunocytochemistry in *Epithelioma papullosum cyprini* (EPC) cell line characterisation.

Results show these techniques to be expeditious and reproducible with cultures providing total DNA and RNA of a quality suitable for molecular analysis of toxic stress.

2.2 Introduction.

Due to increasing awareness of the environment and the subsequent environmental legislative measures, methods for assessing the aquatic environment are diversifying and expanding. Scientists are challenged to meet the needs of those governing authorities, who not only require systems which monitor complex substances but also systems with accurate endpoints, representative of the environmental conditions which persist. With an increasing awareness of ethical concerns relating to live animal experiments, focus on *in vitro* based cell and tissue cultures to deliver alternatives in toxicology studies is increasing.

Unlike primary cell cultures, cell lines often lose traits associated with functional, structural and metabolic properties of the organism from which they are derived (1). Consequently, questions on the usefulness and representative nature of cell lines used in *in vitro* toxicology studies often arise. This is a limiting factor in identifying activated markers representing pollution in the environment.

Specific markers include cytotoxicity, cellular proliferation, functional inhibition, activation of proteins such as HSP (2), genetic damage and alteration in gene expression (3, 4). The toxic effects of chemicals on *in vitro* cell cultures may be assessed using apoptosis, necrosis and expression of proteins associated with genotoxic damage (2,5,6). Other endpoints used to monitor toxic effects include cell morphology, cell attachment, total protein levels and clonogenics (7).

A logical approach when choosing a species for tissue culture development is to select for organisms which traditionally fulfill the role of “indicator species” and/or live in the environment which requires monitoring.

Tissue culture based technologies in aquatic toxicology are typically unreliable with the time and effort required to achieve suitable cultures .

Few established aquatic invertebrate cultures exist to date despite significant effort being directed to this area of research. Ironically much of the pollution, which requires monitoring, is concentrated in environments associated with various invertebrates. In general, the growth of aquatic vertebrate cell cultures is well established. These include Chinook Salmon Embryo (CHSE) cells, *Epithelioma papullosum cyprini* (EPC) cells Blue gill sunfish (BF-2) cells and Rainbow Trout Gonad (RTG-2) cells.

In toxicology studies carried out by Ni Suilleabhain *et al.* 2006, outward migration of cells from explants of rainbow trout was shown to indicate different degrees of water contamination (8). In this study, the explants had to be attached to the culture flask. This is not always achievable and protocols can be laborious. Factors which affect attachment include the organ section in question, surface of the flask, age and condition of the donor animal and often the time of the year. Generally researchers have found varying success depending on the choice of tissue cultured. The literature reveals much success with Rainbow Trout (*Oncorhynchus mykiss*). Tissues from the rainbow trout, which have been cultured to date, include skin, gill, spleen, gonad, kidney, fin, and gut amongst others (9 – 13). In the marine environment, its relative the Atlantic Salmon (*Salmo salar*) is one of the most commonly cultured fish species. However, this species exists in pristine conditions in the wild. Species ideal for eco-toxicological studies should also frequent poorer conditions. The flounder, (*Platichthys flesus*), is a prime candidate species. While there are no reports of *P. flesus* primary tissue cultures to date, Tong *et al*. (1997), have reported culturing a continuous cell line from the gill of the Japanese flounder, (*Paralichthys olivaceus*)

(14). Flounder is a flat fish, which lives in conditions ranging from freshwater to fully saline. It buries itself in the sediment in and around estuaries and ports and other sandy/muddy areas. Hence, it would appear that contaminated water does not inhibit the movement of flounder significantly in such an area. *P. flesus* are relatively easy to capture along the Irish coast using purse seine nets and are also easy to maintain in laboratory conditions. As with many vertebrates, the common tissues cultured from flounder are skin, kidney, spleen and gill.

While the establishment of aquatic vertebrate tissue cultures is generally perceived as being difficult, the generation of aquatic invertebrate species has been elusive, with the exception of the freshwater snail, *Biomphalaria glabrata* (15). It is generally accepted that aquatic invertebrates may be more suitable for eco-toxicological studies, due to their diversity, both in faunal class and feeding type, (i.e. filter and suspension feeders) and their proximity to the sediment.

The blue mussel, *Mytilus edulis* has been used for over 30 years to monitor environmental health conditions by means of the 'Mussel Watch' programme. Their use in toxicology and eco-toxicology has developed rapidly. They are beneficial due to the large volumes of water, which they filter, and their wide geographical distribution (16). Certainly, the development of a mussel cell or tissue culture would be invaluable in progressing the field of eco-toxicology due to the comparative data, which is available.

Few developments have been made in tissue culture of invertebrates. Takeuchi *et al.* (1994) cultured cells from the larvae of *M. edulis galloprovincialis* however these cultures survived for only 2 weeks (17). Le Marrec *et al.* (1995), cultured cells from the heart and gills of the scallop, *Pecten maximus* (18). Mulford and Austin (1998), developed primary cultures from the ovary, testes, hepatopancreas, haematopoietic

tissue, heart, gut, gill, eye-stalk and nerve tissues of the Dublin Bay prawn, *Nephrops norvegicus* (19). A number of different media including L-15, Modified Eagles Medium (MEM) and M199 were used with varying levels of supplements. The best results were achieved with ovary ovarian tissue, which were maintained in 2x L-15 with 5% foetal bovine serum (FBS). One subculture was achieved prior to the collapse of the culture.

Mothersill *et al*. (2000), point out that success with cell culture is closely correlated to the use of young actively growing animals. Indeed, the cell line from *B. glabrata* was derived from the larvae (20). There are many instances of embryonic and larval stages of the life cycle used to generate viable cultures (17 - 18, 21 – 22). However, for various reasons, all of these cultures were relatively short-lived. Developing a cell line from a marine invertebrate remains elusive.

Another impediment to the development of cell and tissue cultures is the difficulty associated with developing and maintaining uncontaminated cultures. Although mainly associated with cell culture, characterisation of the cell may be a factor to consider, particularly in labs where a number of cultures are grown in close proximity as is the norm. Characterisation involves confirming that the cells in the culture are authentic to the tissue of origin and display key markers for their end use. Generally, tissue cultures must retain the characteristics of the cells composing the tissue and characterisation of the cultured cells is critical (20).

Characterisation must confirm that the growing tissue cultures originate from those, which were initially seeded onto the plates. Advances in Polymerase Chain Reaction (PCR) have revolutionised this area. Primers specific to the target species can quickly confirm the origin of tissue cultures. Other techniques used in characterisation include morphology, size, ultra structure, cell-growth kinetics, immunohistochemical

methods, enzyme activity, antigenicity, histochemistry, isoenzyme analysis and chromosome content (23).

One of the main suggestions for the promotion of applications in tissue culture is the possibility of replacing *in vivo* animal testing with *in vitro* tissue culture systems. While this is a worthy cause, it is important that the replacement *in vitro* system is as reliable and relevant. Validation has been defined as the process by which the reliability and relevance of a procedure are established for a specific purpose (24).

In practice, primary cells and tissue cultures are cultured *in vitro* and exposed to a toxic assault. In our approach, we observe mitochondrial (dys)function following toxic assault. The mitochondrion is an organelle of particular interest due to several proteins associated with it, the fact that it has its own genome and the ease at which mitochondria may be extracted from tissue cultures (25). It is also an organelle which may be found highly conserved across species and throughout different trophic levels. Here we report on the application of *in vitro* cultures of fish and shellfish tissues as a platform for the development of eco-toxicological assays based on the availability of DNA and RNA associated with the mitochondrion and mitochondrial proteins.

2.3 Materials and Methods.

Sources of Fish & Shellfish

Rainbow trout (*Oncorhynchus mykiss*) of 200 g average weight were sourced at Rafford Trout Farm, Athenry, Co. Galway. The fish were transported in water tanks and arrived at the laboratory within 4 hours of capture. Flounder were captured with the help of Paul Casburn, Taigde Mara Teo., Carna, Co. Galway. Fish approximately 6cm in length were captured in May and September using the 'purse seine' netting technique along the coast of Galway but mainly at Ballyloughan Strand, Galway. Flounder were maintained in aerated seawater aquaria in the lab.

Blue mussels (*Mytilus edulis*) were sourced at Jasconious Ltd., New Quay, Co. Clare. They were maintained in aerated seawater aquaria in the lab scallop larvae were sourced at Jasconious Ltd., New Quay, Co. Clare. Following spawning and fertilisation, the resulting larvae (at 1st polar body stage) were transported to the lab for subsequent preparations.

Finfish Tissue Culture

Fish were humanely sacrificed according to guidelines approved by the Dublin Institute of Technology Ethics Committee. The number of fish used varied between three and five fish per experiment. Tissues were dissected into small sections (1-2 mm^3) in Petri dishes containing chilled RPMI 1640 growth medium and pooled in preparation for each experiment. These sections were mounted in triplicate for each test group on 25 cm^2 tissue culture flasks (NUNC, Roskilde, Denmark) containing 5 ml RPMI 1640 media supplemented with 12% foetal calf serum, 8% horse serum, 5000 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 0.05 U insulin, 1 ug/ml hydrocortisone, 25 mM HEPES buffer and incubated at 20^{o}C. Each experiment was conducted at least three times and typically five times.

Shellfish Culture

Shellfish were cleaned and swabbed prior to dissection using 70% alcohol. A sterile scalpel was used to prise open the shell and sever the posterior and anterior adductor muscles. Tissues from between three and five fish per experiment were dissected and placed in an antibiotic solution (10,000 U/ml penicillin/streptomycin, 0.01 mg/l kanamycin and 0.04 mg/l gentamycin). Samples were shaken gently for 90 minutes and then repeated using fresh antibiotic solution. Tissue was then cut into 1-2 mm^3 pieces in a Petri-dish containing antibiotic solution and left for a further 90 minutes. The tissue pieces were then mounted in triplicate in 25 cm^2 tissue culture flasks

(NUNC) containing 2x Leibovitz L-15 media (Sigma Aldrich, Dorset, UK) supplemented with 10% foetal calf serum, 10,000 U/ml penicillin / streptomycin, 0.01 mg/l kanamycin, 2 mM glutamine, and incubated at 20^{o}C. Each experiment was conducted at least three times and typically five times.

Larvae Tissue Culture

Pre-conditioned scallop broodstock were placed in seawater warmed to approximately 20^{o}C. Following spawning, the eggs were fertilized using scallop sperm. The resulting larvae were washed, sieved using a 30 μm mesh and placed in fresh seawater at a concentration of approximately 50 larvae / ml. Eighty ml of 'homogenous' solution was removed and sieved/decanted using a 30 μm sieve. Larvae were washed from the sieve into 20 ml sterile seawater (SSW) and allowed to grow for 20 hours at 20^{o}C. 1.5 ml of this (now trochophore larvae) larval stock was removed and briefly centrifuged up to 1000 g. Supernatant was removed and trypsin (0.05%), pronase (0.15%) and collagenase (0.1%) solutions and an equal mix (cocktail) of these solutions were added to the larvae. In control samples, the enzymes were substituted for SSW. Samples were left for 10 mins to dissociate larval cells. Meanwhile, another set of samples were mechanically dissociated by aspirating vigorously through a 1000 μl pipette tip for 1 min.

All samples were briefly centrifuged up to 1000 g to remove excess dissociation fluids. This was repeated twice using SSW to arrest digestion. Finally, the pellet was re-suspended in 1.5 ml of L-15 growth medium. Samples were plated into 2 5cm^{2} flasks (NUNC) and incubated at 20^{o}C. This procedure was repeated every 4 hours.

Tissue Culture Imaging

Photography of tissue cultures was used to monitor cell attachment and growth. Cultures were observed using a Nikon Eclipse E600 Inverted Microscope mounted

with a SPOT RT Color digital camera (Diagnostic Instruments Inc., Michigan, USA) and images processed using SPOT Basic Camera Software (Diagnostic Instruments Inc.)

DNA and RNA Isolation and Visualisation

DNA was isolated from both tissue and cultured cells using the GenElute Mammalian Genomic DNA Kit (Sigma-Aldrich). The DNA was quantified using a BioPhotometer 6131 Spectrophotometer (Eppendorf, Germany) measuring at 260 ηm. Samples were standardised to 10 μg total DNA /ml using molecular grade water for use in PCR studies. RNA was extracted using the Qiagen RNeasy Micro extraction kit (Qiagen, where where) following the manufacturers protocols. Final RNA concentrations were measured spectrophotometrically at 260 ηm and 280 ηm using an BioPhotometer 6131 (Eppendorf, Germany).

Characterisation of Cell Cultures

Forward and reverse primers were designed based on the mitochondrial and nuclear sequences of all fish species investigated. The mitochondrial genome of all species included here is widely available on the internet or in the literature.

The 'Primer 3' primer design program also available on the Internet at the following url:

(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/) was used to generate the unique sequences and designed to have corresponding parameters especially melting temperature and GC ratios. The sequences were synthesised by Sigma-Genosys, UK. Primers, specific to the mitochondrial genome of *Oncorhynchus* spp. (designated RT1) and *Cyprinus carpio* (designated C1 and C2), were designed to confirm the origin of the CHSE-214 and EPC cell lines respectively (Table 2.1). PCR

reaction mixes contained 1 μM each of forward and reverse primer, 10 ng DNA template, 1 x ReddyMix PCR Reaction Mix (Sigma-Aldrich).

Primer Identification	*Sequence*	*Product Size(bp)*
RT	Left Primer - catttagcacttcccatcgc	2569
	Right Primer - tgggctcaaacgataaatcc	
C1	Left Primer - cgtagcccaaacaatttcgt	189
	Right Primer - aatggtgctcggtttgtttc	
C2	Left Primer - acatagccgatcaacgaacc	353
	Right Primer - ctgccacccagtatgtcctt	

Table 2.1 PrimerSequences Selected for Rainbow Trout (*Oncorhynchus mykiss*) (RT1) and Carp (*Cyprinus carpio)* (C1 & C2).

Thermocycling consisted of 2 min at 95^{o}C, 30 cycles of 40sec at 94^{o}C, 2 min at 56^{o}C and 3 min 40 sec at 72^{o}C with a final extension time of 10 min at 72^{o}C using a PTC-225 Peltier Thermo Cycler (MJ Research, MA. USA). Amplified products were separated on a 1% agarose gel and bands visualised using a Gene Genius Bioimaging System (Syngene, UK).

Immunocytochemistry.

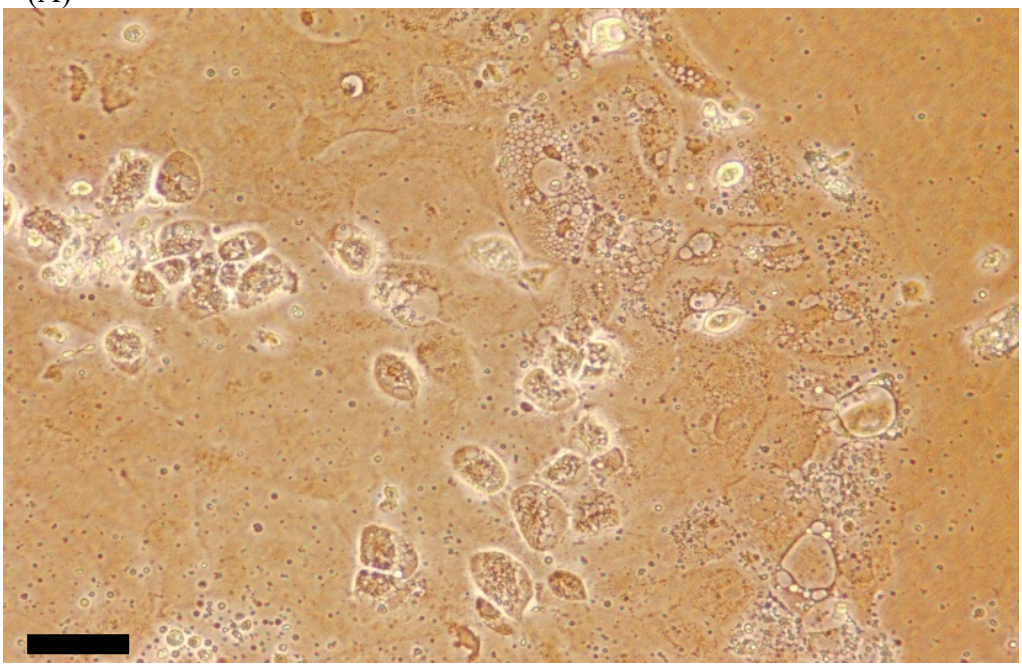
Immunocytochemical analysis was performed using strepavidin peroxidase with a Vectastain ABC kit (Vector Labs, California). Subconfluent cultures of CHSE, EPC, hTERT and HPV-G, cells were grown. hTERT and HPV-G cells were used as positive controls for fibroblast and epithelial cells respectively. All cultures were washed twice in PBS to remove any debris and then fixed in 10% buffered formalin. The primary mouse monoclonal antibody (1:100 dilution), Vimentin (Dako, Denmark) was applied to all cell lines for 1 hour. Simultaneously, primary mouse monoclonal antibody (1:100 dilution), Cytokeratin (Dako, Denmark) was added to a separate suite of cultures for 1 hour. Biotinylated antimouse reagent was then added to all cultures for 30 minutes followed by Strepavidin Peroxidase for a further 30 minutes, with a PBS wash in between each step. The chromagen, 0.02% DAB (Sigma-Aldrich) was then added for 10 minutes in darkness and washed off in distilled water. Cells were then counterstained with Harris haematoxylin and mounted with glycergel. A negative control, where no primary antibody was added was included in each experimental run. Positive staining was observed as a brown staining in the cytoplasm.

2.4 Results.

Growth of Vertebrate Tissues

Primary cultures of gill, spleen and skin from rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*) and flounder (*Platichthys flesus*) were grown *in vitro* (Figure 2.1). Table 2 summarises the species used, the source of the explant and the culture media used. Finfish tissues are least problematic in culture. Skin, gill and spleen are the most commonly grown tissues used in these experiments. Spleen cultures were the slowest growing while skin tended to be the fastest growing tissue. Of the vertebrate species used, *O. mykiss* and *P. flesus* were the most straightforward to culture *in vitro*. Tissues from finfish were culturable and were not affected by contamination as much as shellfish. Gills explant cultures tended to be the most difficult tissues to grow. This was mainly attributed to fungal infections and poor attachment of the explant to the flask.

(A)



(B)

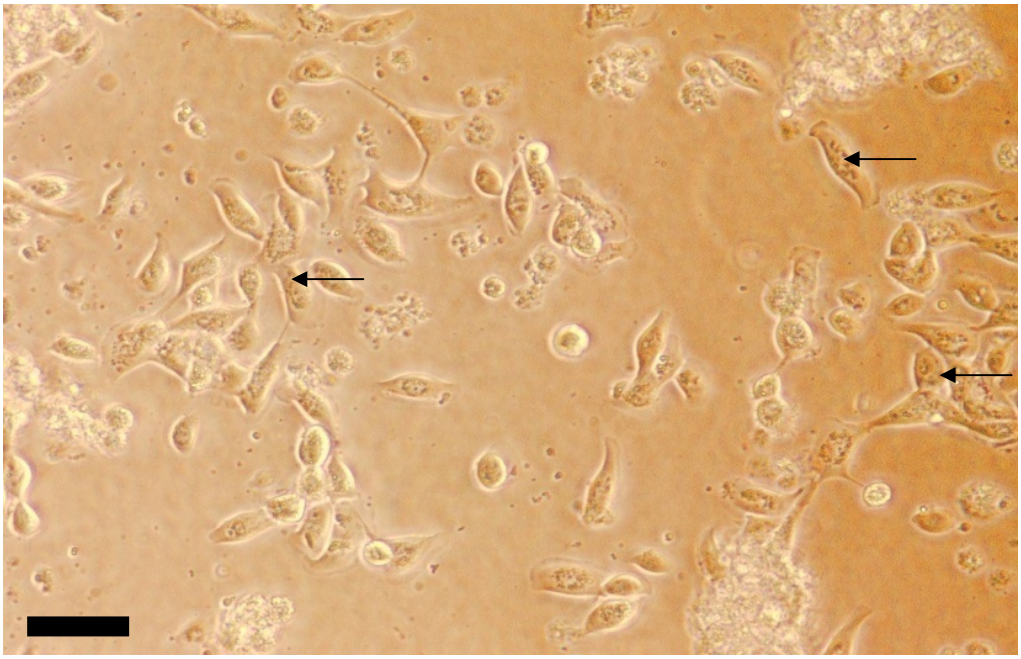


Figure 2.1 Cells migrated from a tissue section of O. mykiss (A) gill and (B) skin after 8 days of incubation.

Identification of cells migrated from gill tissue is difficult due to the lack of distinctive features. Cells migrated from skin tissue are sparse and some cells contain black apoptotic-like bodies (arrows) within the cell cytoplasm. Bar = 50µm.

Species	*Tissue Cultured*		*Media*	*Notes*
Rainbow trout	Skin	Spleen	RPMI	Generally successful. Contamination & poor attachment typical cause of failure in gill
Turbot	Skin	Spleen	RPMI	Gill difficult to culture
Flounder	Skin	Spleen	RPMI	Very good success rate for most organs and particularly skin. Conduit forms from explant.
Clam	Mantle	Pallial Mantle	Leibovitz L-15 (2x)	Generally difficult to grow. Success most likely with P.M. Dissection difficult in smaller organisms. Contamination prevalent.
Mussel	Mantle	Pallial Mantle	Leibovitz L-15 (2x)	High contamination problems. Antibiotic treatment necessary but treatment time affects success.
Scallop	Trochophore Larvae		Leibovitz L-15 (2x)	Dissociation of larval cultures successful but no growth/attachment achieved. Specialist facilities required which is main limiting factor. Also seasonal supply to a certain degree.

Table 2.2 Listing the species cultured in this study, the tissue section used, the media providing best results and general notes on the *in vitro* culture of the specific tissues.

Growth of Invertebrate Tissues

Primary cultures of gill, mantle and pallial mantle have been cultured from blue mussel (*Mytilus edulis*) and Manila clam (*Tapes semi-decussatus*). There were minimal differences in the growth achieved between species or tissue types. Dissection of tissues from the clam was often difficult due to the size of the animal and the nature of the tissue. Tissue cultures of scallop (*Pecten maximus*) larvae, were attempted without success which was indicated by a negative trypan blue exclusion viability test following dissociation.

Growth of Tissue Cultures

Primary cultures from explants of *O. mykiss*, *P. flesus* and *Mytillus edulis* were established on tissue culture flasks and examples are illustrated in Figure 2.1 -2.4. Cells, which have migrated from *O. mykiss* gill following 8 days of incubation in Figure 2.1 (A) were difficult to identify due to the photographic quality and the lack of distinctive features. In Figure 2.1 (B), epithelial cells derived from *O. mykiss* skin emerged. Growth was sparse and some cells contained black apoptotic-like bodies within the cell cytoplasm.

Figure 2.2 (A) and 2.2 (B) are examples of *O. mykiss* and *P. flesus* erythrocyte cells respectively migrated from spleen explants. Generally, there was a progression from these cells to epithelial type cells over time (1 month approx.). Unlike human erythrocytes, fish erythrocytes contain a nucleus, which were easily visible here. In Figure 2.2 (A), 'drumstick' type cells are also evident and have yet to be identified.

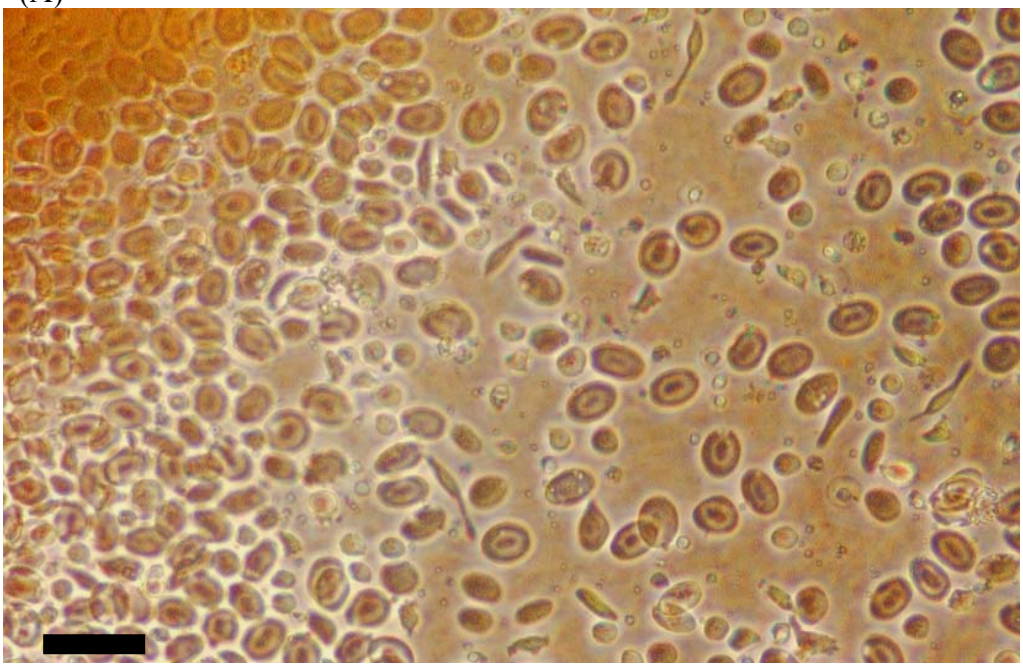
Epithelial cells from the skin of flounder are illustrated in Figure 2.3 (A). Good growth typified tissue explants from this organism. In this photograph, a 'conduit' type structure connects the explant to the outgrowth of cells. Few, if any, cells grew adjacent to the explant but stem from these 'conduits'. Several conduits emerged

along the circumference of each explant. This phenomenon was observed in all successful flounder skin explant cultures.

Figure 2.3 (B) shows epithelial and secretory cells from the pallial mantle of *M. edulis*. Epithelial cells are typified by the finger like projections stretching from the body of the cell while the secretory cells here are rounded in shape and contain many vacuoles.

No obvious effect was observed on the larvae 10 minutes following exposure to this cocktail of enzymes, however by 40 minutes, some cells had dissociated from the larvae and movement of the trochophore larvae was reduced. At 50 minutes post exposure to the enzyme cocktail, the larvae experienced almost complete dissociation however trypan blue exclusion method confirmed the lack of viability at this stage (Figure 2.4).

(A)



(B)

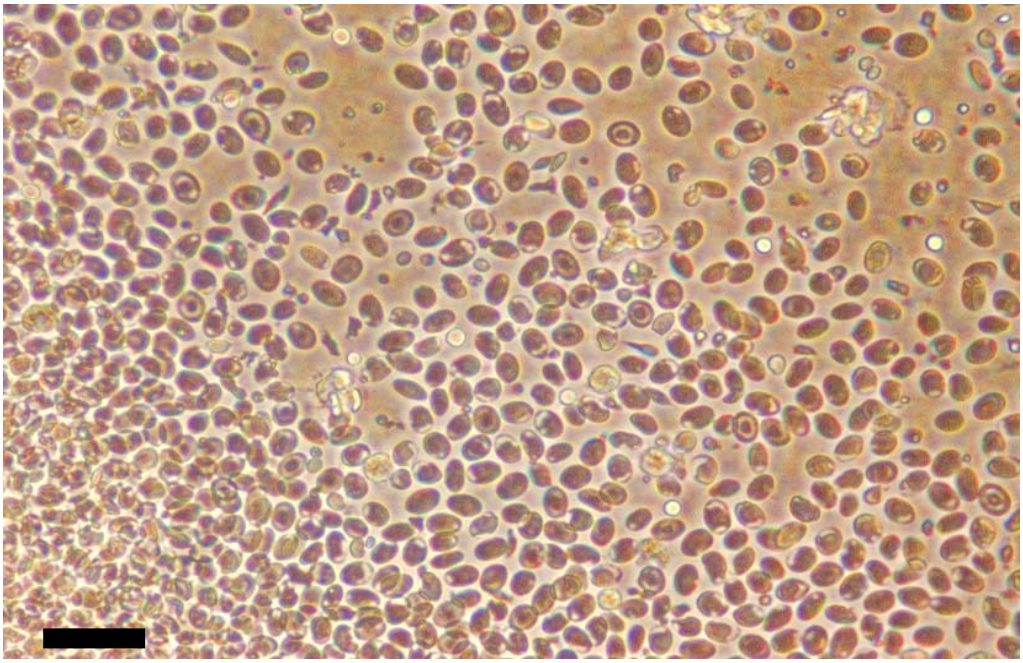


Figure 2.2 Erythrocytes which have migrated from spleen sections of O. mykiss (A) and P. flesus (B) following 8 days of incubation

Bar = 50 µm.

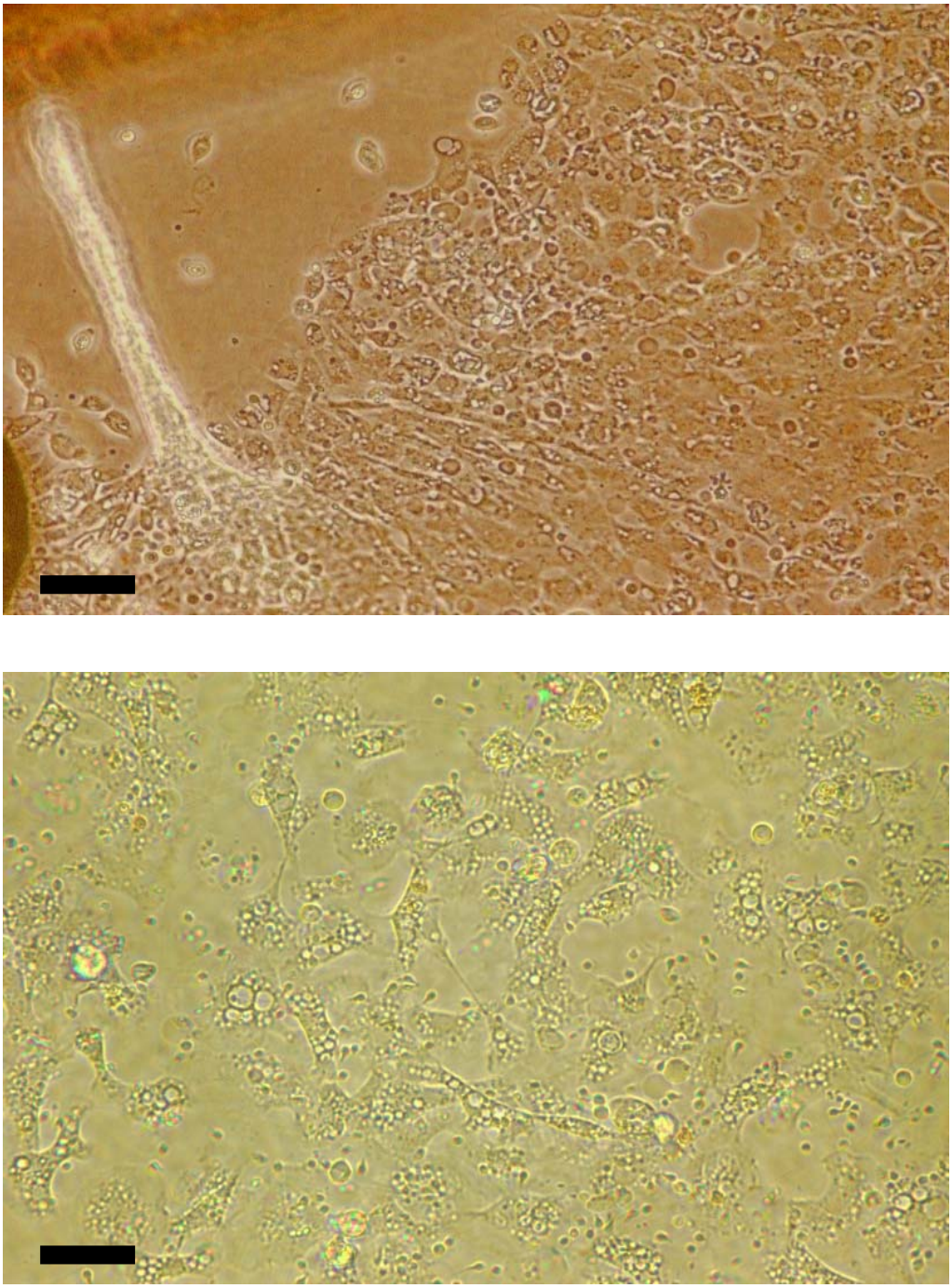
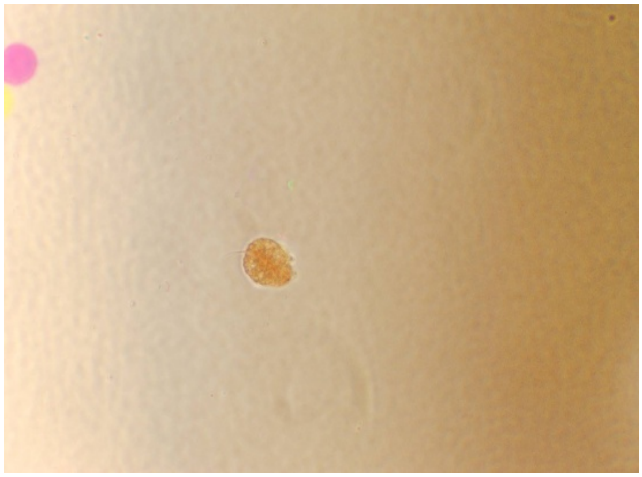


Figure 2.3 Migration of (A) epithelial-like cells from a skin explant of P. flesus (A) arise from 'conduits' connecting the cells to the main explant and (B) epithelial and secretory cells migrating from a section of the pallial mantle of M. edulis.

Images were taken following 8 days incubation. Bar = 50µm.

(A)



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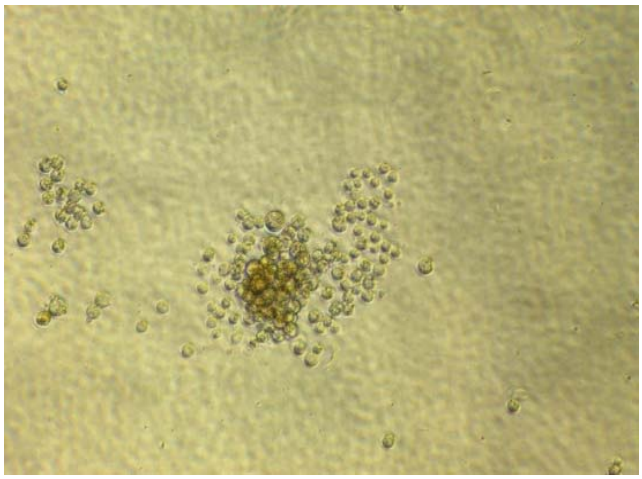

(C)

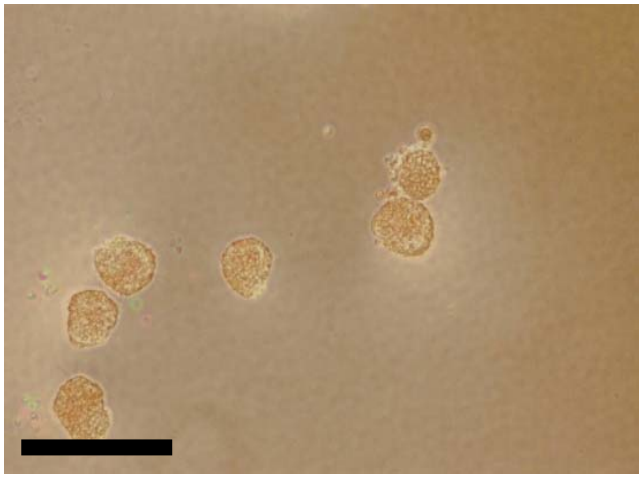

Figure 2.4 A cocktail of Trypsin (0.5%), Pronase (0.15%) and Collagenase (0.1 %) equilibrated to 960 osmol/kg was used in combination to dissociate P. maximus larvae.

Larvae were observed 10 minutes (A), 40 minutes (B) and 50 minutes (C) post treatment (Bar = 500 µm)

Characterisation.

Figure 2.5 shows the results from cell line characterisation using DNA analysis. Bands in lanes 1 and 10 correspond to amplification using salmonid DNA from rainbow trout and the CHSE-214 cells with the RT1 primers set which is specific for salmonids. Analogous bands in lanes 5 - 6 and 14 – 15 confirm the relationship between carp DNA and the EPC cell line. The absence of bands in lanes 2 & 3 containing RT DNA and carp primer sets (-ve control for carp primer sets), lanes 4 containing carp DNA and the RT1 primer set (-ve control for rainbow trout primer sets) and lanes 7-9 containing turbot DNA and the RT1 and carp primer sets confirm that there is no cross reactivity between any of the primer sets and DNA from other target species.

The absence of bands appearing in 11, 12 & 13 also confirm that there is no cross contamination of the cell lines used in this study.

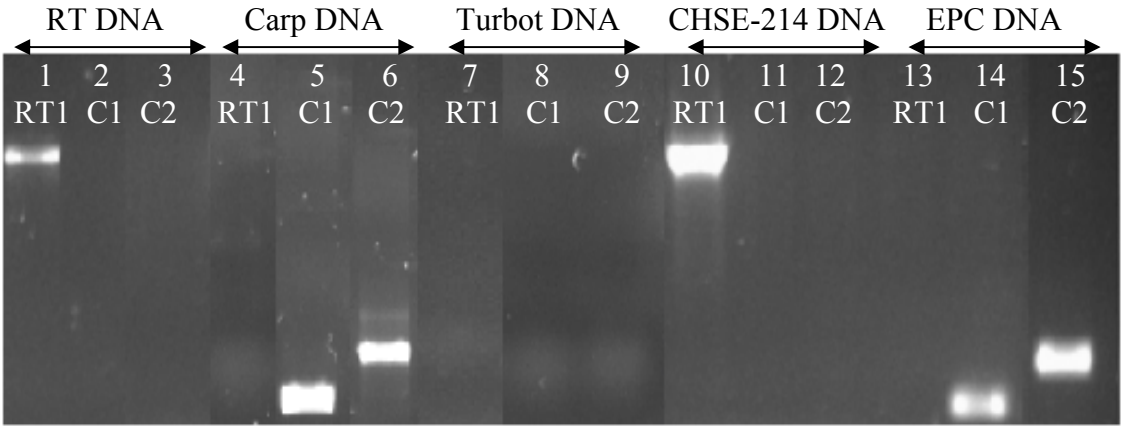

Figure 2.5 PCR amplification is used to characterise the CHSE and EPC cell lines used in this study.

Presence of bands represents a positive reaction with DNA and respective primers. DNA samples were taken from the fish, *O. mykiss* and *C. carpio* and amplified using primer sets RT1 specific for *O. mykiss* (Lane 1) and C1 and C2 specific for *C. carpio* (Lanes 5 & 6). Lanes 2 and3 are negative controls to confirm that no amplification is possible with rainbow trout DNA and carp primers and *vice versa*. Turbot DNA was used as a negative control for all primer sets. DNA was extracted from cell lines and amplified using the same primers. The band appearing in Lane 10 confirms that this DNA does originate from salmonids. The bands found in lanes 14 & 15 confirm that the EPC cell line does originate from carp species.

Immunocytochemistry

CHSE and EPC cells stained with the cytokeratin primary antibody reacted positively as evidenced by the brown staining observed in Figure 2.6 (A) (CHSE-214 data not shown). In contrast, CHSE and EPC cells stained with vimentin did not react positively as shown in Figure 2.6 (B) (CHSE-214 data not shown). As expected, the positive control for epithelial cells (HPVG cells) reacted with cytokeratin and not with vimentin (data not shown), while the fibroblast positive controls (hTERT cells) reacted with vimentin but not with cytokeratin (data not shown). The above suggests that the fish cell lines investigated have epithelial morphology.

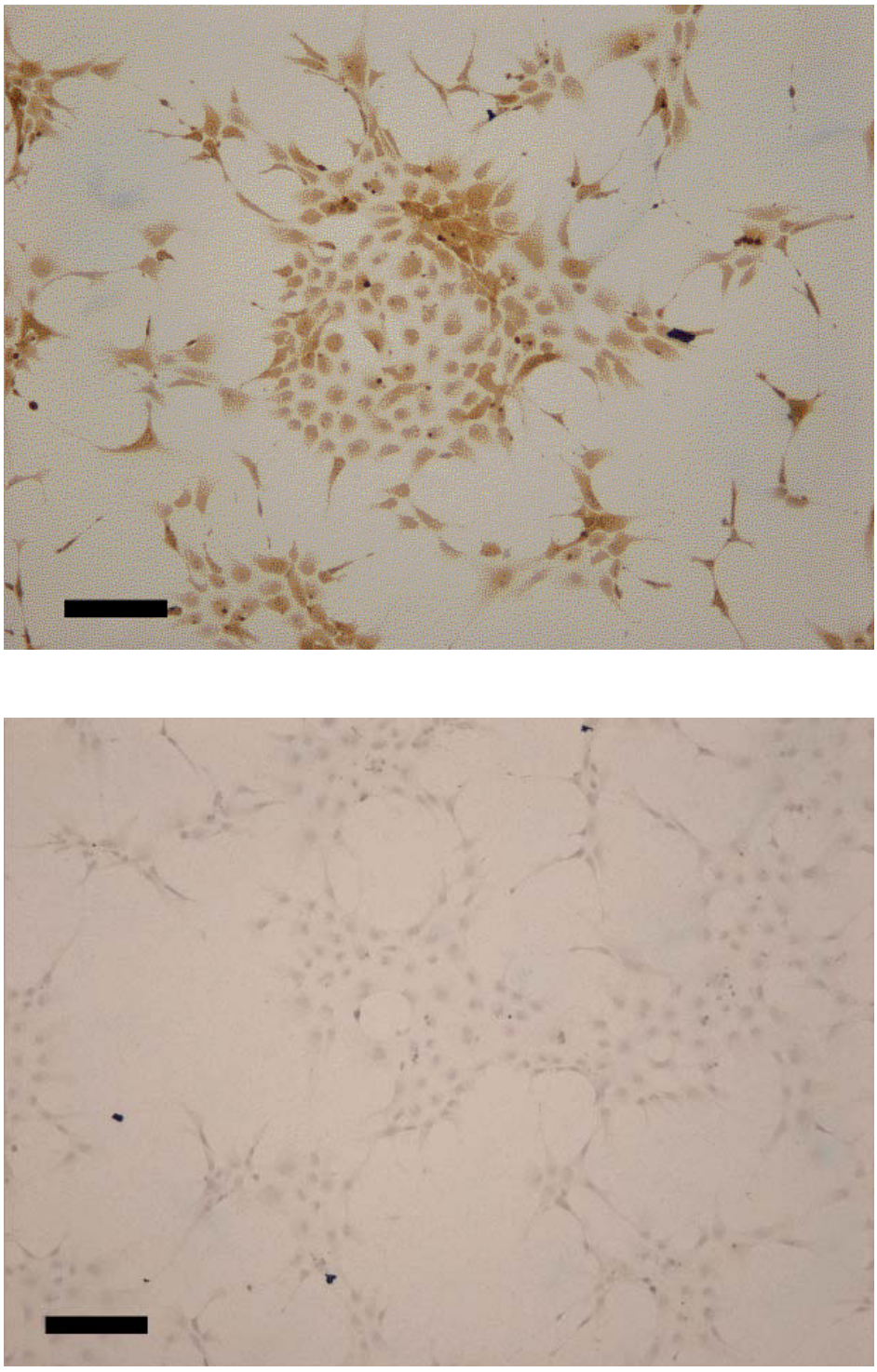

Figure 2.6 EPC cells are stained for cytokeratin (A) and vimentin (B) which are necessary components in cytoskeletal structure associated with epithelial morphology.

Bar = 50 μM

MtDNA amplification.

The amplification of a control region of mtDNA from equal total DNA concentrations shown in Figure 2.7 show variations in the concentration of product generated depending on tissue type, exposure level and time harvested post-exposure. It is clear that amplification is more pronounced (and therefore mtDNA more abundant in the original cell population) in samples derived from gill and spleen DNA than from skin. Indeed there is very little observable difference in the amount of PCR product amplified from DNA found in skin. Generally, there is an increase in the DNA concentration with dose and time of harvesting post irradiation treatment. This is most apparent in the amplification of DNA from spleen samples. Increasing levels of amplified products are also evident in control samples especially in gill and spleen samples and to a lesser degree in skin samples.

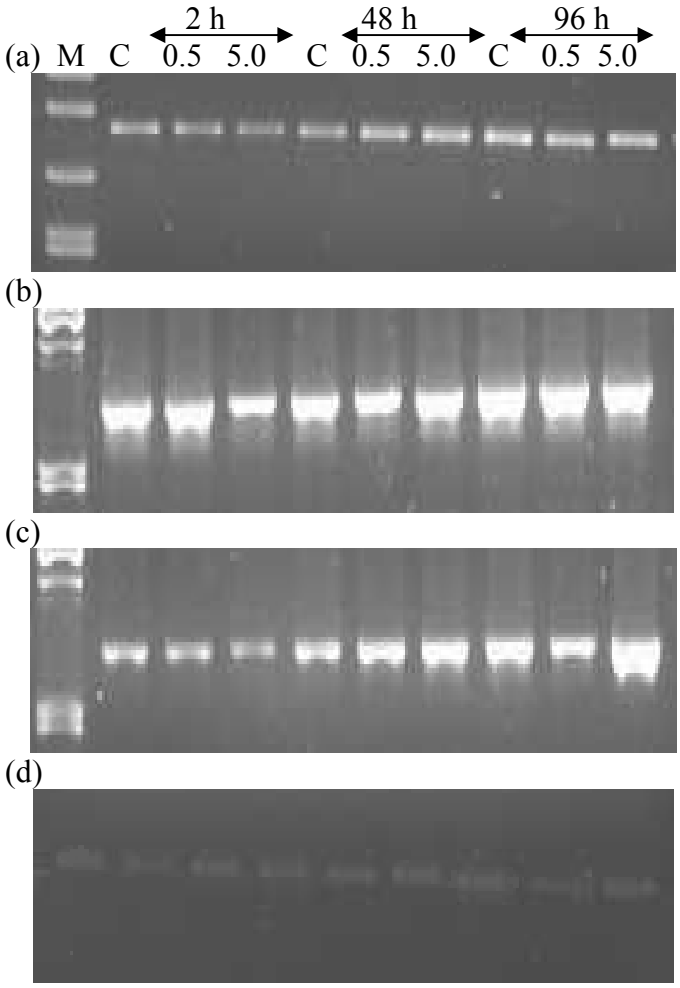

Figure 2.7 Mitochondrial DNA PCR products from rainbow trout (a) skin, (b) gill, and (c) spleen tissues at 2 h, 48 h and 96 h following irradiation at 0.5 Gy and 5.0 Gy and corresponding PCR products from nuclear DNA based RT/DG primer set (d) serving as control.

RNA Quality

Spectrophotometeric analysis of RNA samples (Table 2.3.) gives an indication of the quality of RNA extracted from tissue culture samples. The quality of RNA is determined by calculating the ratio value of absorbance at 260:280. The samples from sections of gill, skin and spleen tissues range from 1.76 – 1.88, 1.75 – 1.88 and 1.81 – 1.90 respectively. Generally, these values are accepted as being that of high quality RNA and suitable for follow up molecular studies.

Treatment	Gill	Skin	Spleen
Control	1.88 ± 0.02	1.83 ± 0.07	1.89 ± 0.03
0.1 Gy	1.87 ± 0.01	1.77 ± 0.04	1.81 ± 0.001
0.5 Gy	1.88 ± 0.03	1.88 ± 0.03	1.90 ± 0.02
1.0 Gy	1.81 ± 0.03	1.78 ± 0.04	1.87 ± 0.01
5.0 Gy	1.76 ± 0.13	1.75 ± 0.05	1.88 ± 0.01
10.0 Gy	1.85 ± 0.02	1.84 ± 0.02	1.90 ± 0.03

Table 2.3 Average *260/280 ratios for RNA extracted from rainbow trout tissues using Qiagen RNA Mini Prep kit.*

2.5 Discussion.

Growth of Tissues

Experiments in tissue growth to date suggest that primary cultures are achievable with most organisms tested. Tissues cultured are listed in Table 2.2. All tissues attempted have been successful in culture with the exception of the scallop (*Pecten maximus*) larval cells. It is difficult to generate quantitative data in primary tissue culture. Results are subject to the authors experience, opinion and discretion. The growth is generally irregular in shape surrounding the explant. The location of the explant may originate from a different region of the organ, even though efforts are made to standardize this, and the animals may be in different states of health or growth cycle prior to dissection. Achieving adequate surface area contact of the tissue to the tissue flask appears to be an important requirement for successful outgrowth. Once the tissue can attach to the flask, outward growth typically follows. Organs such as the gill tend to be particularly difficult to standardize due to their nature and shape. The tissue must have the cut face downward on the flask surface. While this is feasible for skin and spleen explants, gill explants are more difficult to dissect and mount in this way. This may be one reason why gill explants grow inconsistently and attachment is less predictable. The density of gill tissue further compounds these problems given its tendency to float in the culture medium thus delaying or preventing attachment to the flask floor.

Skin explants were less successful when antibiotic washes were used following dissection. Techniques, which avoid using antibiotics in the preparation stages as opposed to the growing stages (i.e. in the medium) were generally more successful. Contamination was more common in invertebrate cultures, most likely due to the

nature of the animals as clams and mussels inhabit difficult environments and as filter feeders, concentrate many microorganisms.

Earlier workers, especially Mulford *et. al.* (2000), showed that the more suitable media for teleosts and shellfish are RPMI and Leibovitz L-15 respectively which concurs with this study. However, Kilemade 2003 (Personal communication) has suggested that growth is also achievable using various mixes of commercially available media. It is important, particularly with invertebrate cultures that the osmolarity of the media are regulated to equilibrate with levels found in the haemolymph of shellfish.

There are several difficulties relating to the culture of invertebrates in particular the contamination problem. The other main difficulty is with the passaging of these cultures. No cultures have been passaged to date. The author considered that media change rather than reseeding on new flasks would be more beneficial in preliminary experiments.

In theory, the targeting of larval cells for the development of viable cell cultures is compelling. Larval cells are at a high growth phase following embryogenesis. Doubling occurs at optimum levels and is visible under magnification. The eggs and sperm may be held in sterile conditions prior to fertilisation and steps involving dissociation may be carried out with minimum effort or treatment. Furthermore, all cells generated from the larvae are stem cells which, some suggest, are easier to grow and may be more suitable for cell line development. There are a number of drawbacks including the difficulty of sourcing and maintaining larvae. Larval culture requires specialised preparation and training. Often, the location of shellfish larvae culture operations are in remote locations making it difficult logistically.

As shown in Table 2.2, growth was not achieved for scallop larval cell cultures. There may be a number of reasons for this. Firstly, this experiment could only be carried out once. Few are in a position to perform a similar experiment given the time, facilities and expertise required. Takeuchi *et al*. (1995) and Le Marrec *et al.* (1995) have carried out similar work on mussels and oysters respectively. More work would be needed to optimise dissociation and growing conditions for the use of larvae in cell culture experiments.

Tissue dissociations using a variety of enzymes (trypsin, pronase and collagenase) in various combinations were used. It may be that dissociation time and enzyme concentration were too extreme. Figure 2.4 shows the effects of a combination of trypsin, pronase and collagenase on scallop larvae over 10, 40 and 50 minutes, where the dissociated cells at 50 minutes were tested for viability and found to be dead. Perhaps a number of extra wash steps following dissociation treatment would have prevented this, or indeed the narrow window of opportunity lay between the 40 and 50 minute time periods.

Contamination was pronounced and steps taken to eliminate this with antibiotic treatment may have been too severe on such early cell stages. Marine invertebrate larvae are notoriously sensitive to low levels of toxin. Certainly both antibiotic treatment and dissociation enzymes are toxic for these organisms. Mechanical dissociation, although prone to contamination is probably the best approach for this reason. Alternative approaches including fertilisation in sterile seawater and subsequent rinsing in sterile seawater prior to culture is recommended. Given that the access to larvae is limited, more resources are required to conduct tissue culture trials with larvae than other species.

Characterisation.

The molecular analysis combined with immunocytochemistry for the characterisation of cell lines was found to be an adequate and convenient approach here. More sophisticated molecular analyses can be performed which could focus these results further if necessary. The combination of both techniques complements each other in that the molecular analyses confirm the organism from which the cells are derived while the staining technique confirms their epithelial nature.

Semi-Quantitative Mitochondrial Genome Frequency Analysis

The effect of radiation on mtDNA transcription as may be observed in Figure 2.7 indicates differences in the initial quality/quantity of DNA. The products generated confirm that these differences are not only associated with the different exposures but also with the different tissue types. The nuclear associated products in Figure 2.7 (d) are low in intensity but constant throughout the tissue types confirming that equal total DNA was added to each PCR reaction. In contrast, the amplified bands in the treated gill and spleen samples are more intense than in skin. The distinct increase in band intensity with increasing dose in gill and spleen indicates an increase in the proportion of mtDNA in the starting sample. However this increase in intensity is not found in the skin samples indicating no effect of radiation on that organ. This concurs with earlier work which shows that mitochondria in skin tissues and the skin tissue in general is least sensitive in comparison to the other two tissues (26). The increase in the levels of amplified products in the control samples over time suggests that the disturbance of the tissues associated with these experiments and the culture *in vitro* initiates an increase in mitochondria production even in non-irradiated samples.

RNA Quality

We extracted RNA in this study so as develop a gene expression assay to identify toxic stress, in this case from γ radiation. Fundamental to the development of such

assay, is the ability to isolate quality RNA from the cultured tissue samples. The average 260/280 ratios shown in Table 2.3 suggest that the quality of the RNA range between 1.75 and 1.90 for gill, skin and spleen tissues. It is generally accepted that an OD $\geq$ 1.8 and $\leq$ 2.0 is indicative of good quality RNA. The fact that good quality RNA can be retrieved from tissues which are subjected to *in vitro* tissue culture followed by radiation exposure is a reflection of the robustness of RNA in this case.

To conclude, the development of *in vitro* fish and shellfish models for use in aquatic eco-toxicology studies was a worthy exercise however several limitations and barriers to their development persist. Among these, the development of reliable cultures of both cell lines and tissue cultures is the primary. While cell lines are easily characterised and offer many opportunities in the field of eco-toxicology, presenting applications in areas such as spectroscopy and flow cytometry, the literature suggests that tissue cultures are more true to their original structure, function and genetics. While we demonstrate that tissue cultures can be initiated and are sustainable *in vitro,* for periods adequate for bioassay, many can be unreliable with some tissues not attaching to the flasks for a variety of reasons. In this study we show that the establishment of attached tissue cultures need not be necessary for the development of bioassays and alternatively, demonstrate that the tissue culture aspect can facilitate a 'holding and treatment' facility rather than a bioassay in itself. We show that quality DNA and RNA can be isolated from tissue cultures following this approach for further molecular analyses such as mitochondrial sequencing and associated gene expression studies. Consistently successful tissue cultures were achieved from rainbow trout, flounder and to a lesser degree mussel. Furthermore, the use of molecular techniques, coupled with immunocytochemistry techniques combine well to confirm identity and characterise cells in culture.

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CHAPTER 3 ASSESSING PUTATIVE MITOCHONDRIAL BIOMARKERS OF ECOTOXICOLOGY USING γ RADIATION AS A TOXICANT MODEL *IN VITRO.*

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4 Figures, 2 Tables.

The mitochondrion as a biomarker of radiation-induced damage.

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3.1 Abstract.

O'Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., Lyng, F.M., McClean, B. and Murphy, J.E.J. Assessing the mitochondrion as a biomarker of fish tissue damage using γ radiation as a stress model *in vitro*.

There is an ever-increasing need for biomarkers to identify toxic stress in the aquatic environment. Such techniques need to be accurate, expeditious, ethical and economical. Typically, *in vitro* based platforms fit these criteria however many of these systems often undergo 'assay drift' and consequently do not fully represent the real-life situation.

In recent years, there has been growing interest in the mitochondrion and its (dys)function or altered function and dynamics as a marker of toxic assault. The mitochondrion is an essential organelle in the cell and is associated with energy production and metabolism in the organism as it is the site of oxidative phosphorylation (OXPHOS). It has its own genetic material which is more susceptible to damage than nuclear DNA (nDNA) due to its proximity to the site of OXPHOS, the absence of introns, the lack of a protective histone coat and effective repair mechanisms which are present in nDNA. Stress responses including increases in mitochondrial mass and alteration in the activity of proteins associated with OXPHOS have been reported and offer potential as putative biomarkers of toxicity.

In this study, we used real-time PCR to identify alterations in mitochondrial genome copy number in cultured fish tissues exposed to γ radiation. These values were compared to the activity of the citrate synthase enzyme, an established marker of mitochondrial mass in cells. Results show that while this approach is appropriate and the technique is robust, expeditious and straightforward, further development is required to yield greater enhancement and sensitivity.

3.2 Introduction.

Developing environmental biomarkers systems which are expeditious, accurate, economical and ethical continues to be a huge area of interest in the scientific community. This is mainly due to continuous amendments to legislation, greater complexity of pollutants and increased pressure on the aquatic environment. While *in vitro* systems, based on cell lines, would appear to satisfy many of the above criteria, they can be susceptible to 'assay drift' (1 - 3). Hence these may not truly represent the trophic structures and complexities which exist in the aquatic environment and which to date, had been served well by batteries of live organisms representing a wide range of trophic levels.

Aside from the economic cost of maintaining the large numbers of organisms required for meaningful results, the ethical and legislative considerations associated with live animal experiments further incentivises the development of viable alternatives. Many molecular scientists are targeting functions and cellular processes in various organisms which identify toxic stress. Several have identified the mitochondrion as a potential candidate for this. The mitochondrion is found in almost all cells of eukaryotic species including plants and animals. It is a polymorphic organelle responsible for the majority of energy production in the cell. The mitochondrion has its own genome and is able to replicate, transcribe and translate its own DNA independent of the nDNA. However, mitochondrial and cellular functions are interdependent. The mitochondrial genome has the translational capabilities for 13 of the 87 proteins necessary for OXPHOS. Other proteins necessary for this function are synthesised in the cytosol and imported into the mitochondrion (4, 5).

More recently, the importance of the mitochondrion in other functions within the cell including its central role in apoptosis has been recognised (6). Energy is stored in

cells by means of high-energy phosphate bonds, formed in molecules of adenosine tri phosphate (ATP). ATP is converted from adenosine diphosphate (ADP) by the addition of a phosphate group. This occurs anaerobically in the cell cytoplasm via glycolysis and more efficiently through the aerobic process of OXPHOS in the matrix of the mitochondrion (7, 8).

Irregular mitochondrial function and/or dysfunction and mtDNA damage is associated with aging, age-related disorders, cancer and a growing number of other common metabolic and neurological disorders (9-11). While many of these disorders are transferred genetically through the maternal lineage, many others are associated with dysfunction following exposure to toxins. The mitochondrial genome has no protective histone coat and the mtDNA polymerase γ has no proof reading capability polymerases associated with nDNA (12). This is compounded by the absence of introns and proximity to free radical production (13).

Mitochondria and more specifically mtDNA are typically heteroplasmic. The organelle contains several copies of its genomic DNA which are often not identical to each other, as one would expect. Typically the degree of heteroplasmy is an indicator of the damage of some toxic event past or present. Heteroplasmy enables the mitochondria to sustain normal function until a threshold level of mutation is reached. Hayashi *et al*., (1991), demonstrated that the threshold level for mutant DNA is 60% and this is sufficient to inhibit overall mitochondrial function (14). There are tissue specific differences in the threshold levels which can exist without clinical effects emerging (15, 16). Tissues with higher energy demand such as muscle and nervous tissue have a lower threshold level and explains why most mitochondrial diseases occur or present first in these tissues.

It is therefore becoming apparent, that while mitochondrial (dys)function may be studied to identify associations with disease in humans, they may also be utilised as biomarkers to indicate toxic stress in other organisms (17, 18).

The mitochondria react quickly to changing energy demands within a cell and support the organ by increasing in number, increasing IMM folding and increasing OXPHOS turnover (4). Mitochondrial biogenesis is controlled by at least two significant synchronized signalling mechanisms. Firstly, increases in the levels of calcium in the cell activates the calcium-calmodulin kinase (CAMK) enzyme which is associated with the expression of proteins necessary for mitochondrial biogenesis including NRF-1, NRF-2, PGC-1 and mitochondrial transcription factor A (mtTFA) (19). Secondly, a reduction in the cellular concentration of high energy phosphates including ATP and phosphocreatine are necessary for mitochondrial biogenesis (20). This is linked to the activation of 5′-AMP activated protein kinase (AMPK), an enzyme closely related to CAMK which stimulates the other proteins necessary for biogenesis (21, 22). Indeed Lyng *et al.* (2006) identified calcium flux in cells exposed to irradiated cell conditioned medium (ICCM) in HPV-G cells within 30 min (23).

Notably, mitochondria have been reported to modify their activity and position within the cell based on energy requirements or exposure to stress including radiation, toxins and hypoxic conditions (21, 24 - 26).

It has also been reported that mitochondrial numbers can be increased by radiation (27, 28). More recently, Nugent *et al*. (29) identified increased levels of mitochondrial activity following exposure to both direct and bystander medium in human cell lines

In this study, we hypothesise that alterations in the numbers of mtDNA relative to nDNA can be used to identify exposure to toxic stress in fish tissues. In an effort to

develop a bioassay based on altered mtDNA levels, we excised sections of rainbow trout, *Oncorhynchus mykiss* gill, spleen and skin tissue and examined the ratio of mtDNA nDNA in control and irradiated sections using real time PCR. Results are compared to relative mitochondrial mass levels.

3.3 Materials and Methods.

Rainbow trout (*Oncorhynchus mykiss*) of ≅ 200 g average weight were collected at a Rafford commercial trout hatchery in Galway, Ireland. Fish were sacrificed in accordance with guidelines approved by the Dublin Institute of Technology Ethics Committee, i.e. involving an overdose of anesthesia.

Tissue Explant Preparation and Irradiation

Gill, skin and spleen tissue explants were excised from rainbow trout immediately post mortem. Tissue sections were mounted in T-25 tissue culture flasks (Sarstedt, Germany) containing 5 ml MEM (Minimum Essential Medium) (Sigma-Aldrich) supplemented with 10% (v/v) foetal calf serum (Gibco Biocult, Scotland), 2 m*M* L-glutamine (Gibco Biocult), 40 IU/ml penicillin/streptomycin (Gibco Biocult), 30 m*M* Hepes buffer (Gibco Biocult), and 1% (v/v) non-essential amino acids (Gibco Biocult). Flasks were incubated overnight at 20^{o}C to allow explant attachment. Explants were then exposed to either 0 Gy, 0.5 Gy or 5 Gy radiation from a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin 6, Ireland) using a dose rate of 1.8 Gy/min at a source-to-flask distance of 80cm. Explants were removed from the flasks 2hr, 48hr or 96hr post irradiation and stored at -20^{o}C.

DNA Isolation, Quantification and Analysis

DNA was isolated from fish tissues using the GenElute Mammalian Genomic DNA Kit (Sigma Aldrich). The DNA was quantified to confirm adequate yield using a Biotech Ultraspec 3000 UV/Visible spectrophotometer (Pharmacia, Stockholm)

measuring at 260 nm. The 260 nm:280 nm ratio was also measured to confirm DNA quality. Samples were then standardised to 10 μg/ml using molecular grade water.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Restriction fragment length polymorphism (RFLP) analysis was carried out on PCR products RT1 – RT7 and RTD1. These primer sets had been designed and confirmed to amplify the entire rainbow trout mitochondrial genome (Table 1). RFLP analysis can detect differences in amplified homologous sections of DNA based on size differences when these sections are cut using restriction enzymes. The sequences generated by the primer sets were entered into the NEBcutter restriction mapping program (30) together with a list of restriction enzymes available in the laboratory Restriction digests consisted of;-

PCR Product Sample	10 μl
Restriction Enzyme	1 μl
Appropriate Buffer	3 μl
Dist. Sterile H_2O	6 μl

The samples were incubated at 37^{o}C for 20 h. Restricted bands were visualised using the Gene Genius Bioimaging System (Syngene, UK).

Primer	Sequence Origin	Start bp	Prod. Size (bp)	Sequence
RT 1 (F)	Rainbow trout mitochondria	437		atccttaagaaaccagcccc
RT 1 (R)	‘’	3031	2595	gttttgatttgcaagaggcg
RT 2 (F)	‘’	2338		aactttagtttaggcccccg
RT 2 (R)	‘’	4923	2586	atctcttgaggatgggttcg
RT 3 (F)	‘’	4768		catttagcacttcccatcgc
RT 3 (R)	‘’	7336	2569	tgggctcaaacgataaatcc
RT 4 (F)	‘’	7170		ttttgattcttcagccaccc
RT 4 (R)	‘’	9729	2560	aggcttgaatcatggctacg
RT 5 (F)	‘’	9546		attagccttttatccgccc
RT 5 (R)	‘’	12138	2593	aggccaaaacaataaagggg
RT 6 (F)	‘’	11920		aactgtgatgagctgcctgc
RT 6 (R)	‘’	14518	2599	gaagttatgtagtgtaagggttggg
RT 7 (F)	‘’	14355		cctcaaacttcctacccacc
RT 7 (R)	‘’	276	2564	ggagcccgtgttaattgg
RT 1A	RT Mito D-loop	16559		accctccctagtgctcagcgagagg
RT 1B	1-1003bp	1020	1104	agttaagctacgtcagcggcgg

Table 3.1 Primers for amplicons used in RFLP analysis of the rainbow trout mitochondrial genome, the starting location and the amplified product size expected. The combined products amplified by these primers will generate the entire mitochondrial genome of rainbow trout.

Single Strand Conformational Polymorphism (SSCP) Analysis

Single Strand Conformational Polymorphism (SSCP) analysis was carried out on products amplified which comprise the entire mitochondrial genome. Following restriction of the products as described above , an equal volume of SSCP loading buffer (95% Formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.1% bromophenol blue, adjusted to pH 7 using NaOH) was added to the restricted products in an thin-walled Eppendorf tube.

The DNA samples were then denatured into single stranded products by boiling for 10 min. The samples were immediately placed in ice to cool quickly. This prevented the DNA from re-annealing into double stranded product. The samples were then held at –20^{o}C until required for running on a polyacrylamide gel.

The polyacrylamide gel solution was prepared using 20 ml of 30% acrylamide, 7.5 ml 10X TBE, 40 ml sterile distilled water, 7.5ml 50% glycerol and 500 µl ammonium persulphate (NH_4SO_4). Glass plates with 3mm spacer bars and a comb were cleaned thoroughly, assembled and clamped together. 1% agarose was used to seal along the outside of the spacer bars to avoid any leakage of polyacrylamide. Just prior to pouring the gel solution 62.5 µl of TEMED was added. The gel was submerged in 1x TBE buffer prior to removing comb leaving intact wells in place. Fifteen µl of each of the single strand samples containing loading buffer were loaded into each well. The gel was run overnight (~16 h) at 16 mA.

To develop the gel, impregnation solution (1g $AgNO_3$, 1.5 ml 37% HCOH, made up to 1l with ddH_2O), developing solution (30 g Na_2CO_3, 1.5 ml 37% HCOH, 2 mg $Na_2S_2O_2$ made up to 1l in ddH_2O) and acetic acid (10%) solution were prepared and chilled on ice. The gel was placed in a shallow tray containing acetic acid and agitated for 20 min to fix. The gel was then and placed in ice cold impregnation

solution for 30 min. The gel was briefly rinsed and placed in developing solution until bands appeared (~5 min). The gel was then fixed by immersion and agitation in acetic acid for 2-3 min prior to visualisation and documenting using the Gene Genius Bioimaging System.

Real-Time PCR

Table 2 shows the details of all primers used in this study. The efficiency of each primer set (E) was determined by inclusion of a dilution series of template DNA in a PCR reaction containing 1x SYBR Green Master Mix (Qiagen, Germany) and 0.5 μM of both forward and reverse primers [Table 2]. An mtDNA and an nDNA primer set are used in the study to identify the relative levels of mitochondrial to nuclear genomes following toxic assault.

Primer	Amplicon (bp)	Efficiency	Sequence
RT5 Fwd	183		attagccttttatccgccc
RT4 Rev		1.92	aggcttgaatcatggctacg
RT18S Fwd	285		gttccgaccataaacgatgc
RT18S Rev		2.10	gctccaccaactaagaacgc

Table 3.2 The primers used in this study for the amplification of mitochondrial and nuclear DNA show the amplicon size and the level of efficiency of the reaction using real time PCR.

Relative expression values were calculated following the mathematical expression $[E_{Nuc}^{(Ct\ Nuc)} / E_{Mito}^{(Ct\ Mito)}] \times 4$. This equation was formulated to express the number of mitochondrial genome copies relative to nuclear genomes in tetraploid species such as salmonids, and to which rainbow trout belong.

Citrate Synthase Analysis

170 µl of 10 mM TRIS Buffer, 2 µl 15 mg/ml Acetyl CoA, (20 µl 2 mg/ml 5'5'-dithio-bis-(2-nitrobenzoic acid (DTNB) and 5 µl tissue homogenate were equilibrated to 30 ^{o}C. 5 µl 10 µg/ µl Oxaloacetic acid was added and the rate of absorbance change recorded at 412 nm for 5 min in a temperature controlled Helios scanning spectrophotometer (Thermo Scientific, MA, USA). Protein concentration of each sample was quantified using the Bradford Assay (Bradford, 1976). Citrate synthase (CS) activity was calculated as nmoles/min/mg protein using the equation A = ecl ($\varepsilon_{412} = 13.6\ nM^{-1}\ cm^{-1}$) to convert ΔA/min to nmoles per min.

Statistical Analyses

Data are presented as the mean values ± standard errors of three independent experiments incorporating at least three replicates per experiment. All statistical analyses were carried out using the SigmaStat software package (SPSS Inc.). Significance was determined using the Student's *t*-test and differences were considered significant if $p \leq 0.05$.

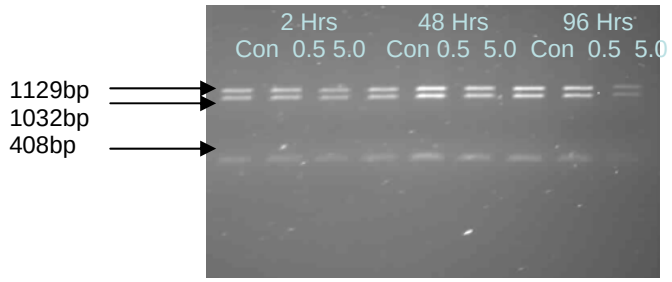
3.4 Results

Restriction Fragment Length Polymorphism (RFLP) Analysis

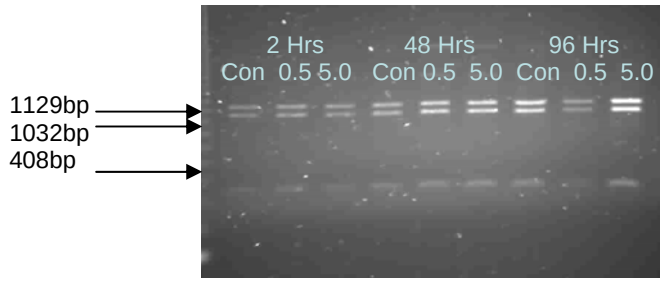
Products amplified, using the mtDNA based RT3 primer set are shown in Figure 1 below as a representative of all RFLP results. Here, the RT3 amplicons for each tissue type were restricted using Ava I endonuclease to identify if mutations in the mitochondrial genome of rainbow trout following radiation could be identified. This

was carried out on DNA samples isolated from control and irradiated tissue explants cultures of skin, gill and spleen. Restricted products of 1129 bp, 1032 bp and 408 bp shown in the gels appear to be similar in size and intact, indicating either the absence of any damage to this region of the mtDNA or the inapplicability of this technique.

(a)



(b)



(c)

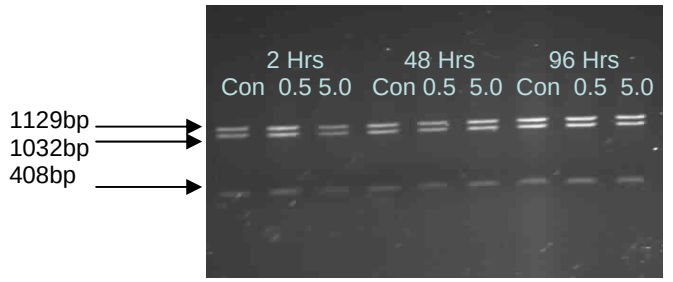


Figure 3.11RFLP analysis of the RT3 product amplified from (a) gill, (b) skin and (c) spleen tissue cultures of rainbow trout and restricted using AVA 1 restriction enzyme into 1129bp, 1032bp and 408bp fragments. Lanes 1-9 represent treatments at 0 Gy, 0.5 Gy and 5 Gy at 2 hours, 48 hours and 96 hours respectively.

Single Strand Conformational Polymorphism (SSCP) Analysis

The banding in gels shown in Figure 2 represents single strand DNA sections. The gels in shown Figure 2 (a), (b) and (c) do not identify any polymorphisms, and are representative of the SSCP analyses carried out on the rainbow trout tissue culture samples. There are no obvious polymorphisms, indicative of damage to the mtDNA in the irradiated tissues.

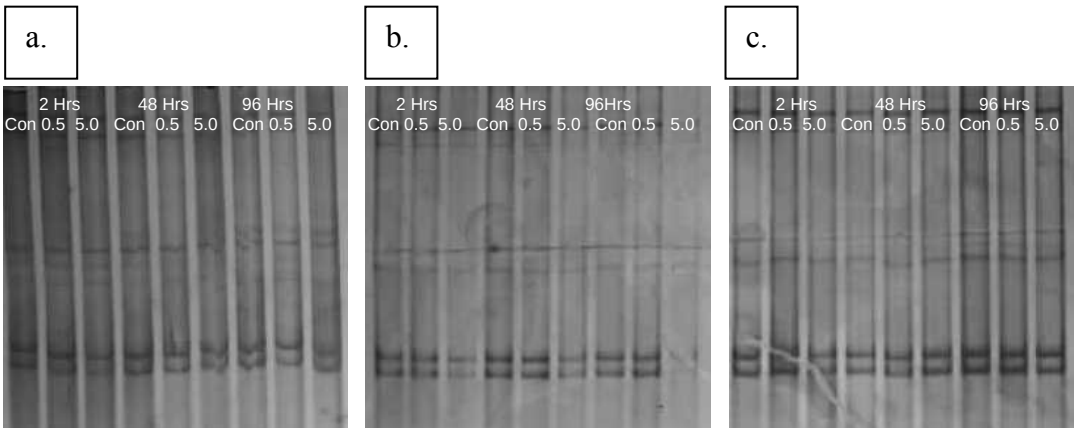


Figure 3.2 Polyacrylamide gels with banding for single strand conformational analysis of rainbow trout (a) gill, (b) skin and (c) spleen tissue cultures following exposure to radiation. Lanes 1-9 represent treatments at 0 Gy, 0.5 Gy and 5 Gy at 2 hours, 48 hours and 96 hours respectively.

Real-time PCR

Real time PCR determined the number of mitochondrial genomes per cell. Generally, gill and skin tissues had a greater number of mitochondrial genomes per cell than spleen. The highest relative copy number (28.9) of mitochondrial genomes per cell was found in gill tissues exposed to 5 Gy and sampled 48 hours post exposure. Conversely, the lowest relative copy number (2.2) was observed in skin tissues 2 hours post exposure to 0.5 Gy dose [Figure 3].

The relative copy number of mitochondrial to nuclear genomes in each tissue type varied considerably depending on exposure and time of analysis post exposure. Control samples from all tissues vary in levels of mitochondria relative to nuclear over time. There was a general increase in the relative copy number found in control samples from the 2 hour time point to the 48 hour time point. Little change was observed in the relative copy number in control samples between 48 hour and the 96 hour time point.

There was a significant increase in the relative mitochondrial levels in gill at 2 hours with increasing dose. If incubated for 48 hours, this increase was only maintained in the 5 Gy dose with no change at the 0.5 Gy dose. If incubated for 96 hours, this effect became more prominent with a significant decrease in mitochondrial genomes at the 0.5 Gy dose and a significant increase at the 5.0 Gy dose.

In skin, this effect was observed at the 2 hour time point. The 0.5 Gy dose exhibits a significant decrease in mitochondrial genomes and the 5.0 Gy dose a significant increase following the treatment. There are significant decreases in the relative mitochondrial numbers in skin tissues at 48 hours and 96 hours when compared to controls.

There is only one significant change in the relative copy number of genomes in spleen tissues. This increase occurs after incubation at 48 hours in tissues exposed to 5 Gy radiation.

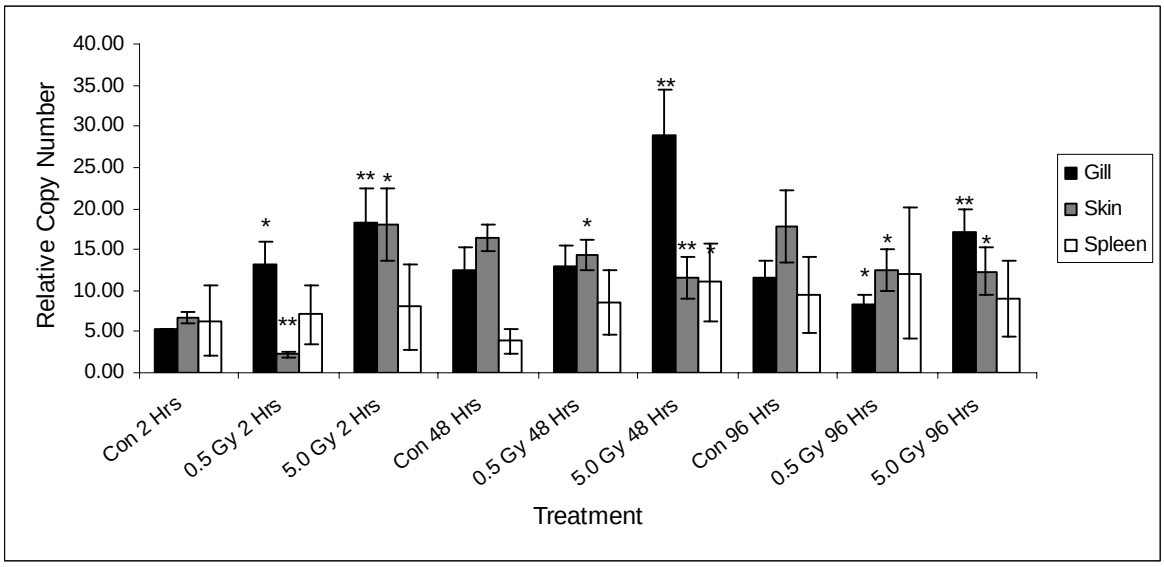


Figure 3.3 Real time PCR results showing the relative copy number of mtDNA genomes per cell in rainbow trout gill, skin and spleen tissues exposed to 0 Gy, 0.5 Gy and 5.0 Gy radiation and incubated for 2 hours, 48 hours and 96 hours following the radiation exposure. The calculations are based on the equation outlined in Pfaffl (2001) [$E_{Nuc}^{(Ct\ Nuc)}$ / $E_{Mito}^{(Ct\ Mito)}$] x 4 and multiplied by four to account for the tetraploid nature of rainbow trout. * denotes $P \leq 0.05$, ** denotes $P \leq 0.005$

Citrate synthase activity is generally higher in gill and skin tissues than in spleen tissues [Figure 4]. The highest level of CS was recorded in skin tissues exposed to 0.5 Gy radiation and analysed 48 hours following this exposure. Conversely, the lowest level was observed in the control spleen samples for the 96 hours post exposure treatments.

While CS data generally correlates with the data found in the relative copy number of genome data for these tissues, few correlations exist between both sets of data.

An exception to this was observed in gill tissues analysed 48 hours post exposure. Here, a significant increase in CS and relative mitochondrial genome copy numbers correlate. Generally, there is a decreasing trend of CS activity in all tissues from 2 h post exposure to 96 hours post-exposure.

In gill samples 2 hours post exposure, CS levels decreased significantly following a 0.5 Gy exposure. Conversely, at the 48 hours time point post exposure, a significant increase in CS level was observed in the 5.0 Gy samples. 96 hours post exposure, a dose response-type effect was observed with significant increases in CS levels following 0.5 Gy and 5.0 Gy exposures.

The skin samples exhibited a converse effect. There was a significant increase in the level of CS in skin exposed to 5.0 Gy 2 hours post treatment. There was also a significant increase in CS in the samples exposed to 0.5 Gy 48 hours post exposure.

The spleen samples exhibited the a dose dependent response type trend with increasing CS levels at both 2 hours and 96 hours following exposures. There was no change of CS levels in spleen tissues at 48 hours.

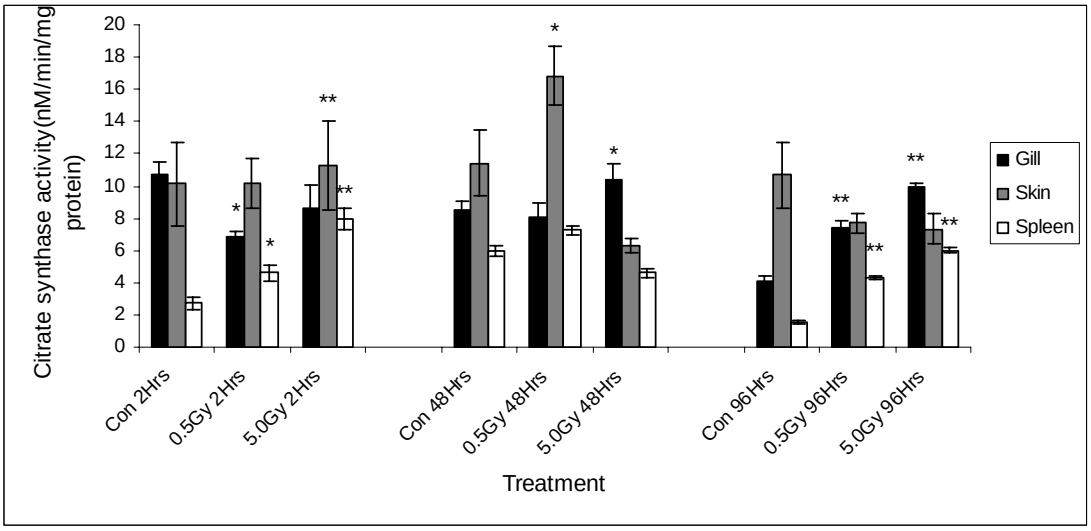


Figure 3.4 Citrate Synthase activity levels found in fish gill, skin and spleen tissue cultures exposed to radiation at 0 Gy, 0.5 Gy and 5.0 Gy gamma irradiation and analysed 2 hours, 48 hours and 96 hours post exposure.

3.5 Discussion

The mitochondrion, given its significance within the cell is theoretically an ideal candidate for applications in the area of biomarker development. It is a distinct organelle associated with the majority of energy production within the cell. While it is only responsible for 13 of the estimated 87 proteins necessary for OXPHOS, any functional defects within the mitochondrial fraction elicit detrimental effects in the OXPHOS process. Furthermore, the phenomenon whereby toxic exposure increases the level of mitochondrial activity can be assessed using PCR technologies. Equally, by focusing on an organelle as ubiquitous as the mitochondrion, biomarker systems based on tissue culture platforms of a vast range of species can be utilized. This facilitates the reduction of live animal experiments by increasing the number of sections which can be assessed for mitochondrial dysfunction following toxic assault.

In this study, analysis of the entire mitochondrial genome is carried out using primers to amplify consecutive sections of DNA. In theory, RFLP analysis should show differences in size based on mutations occurring in the double stranded mitochondrial DNA. However, in this study, no such mutations or size differences existed indicating that either radiation does not adversely affect DNA in the fish tissue sections tested here or that the technique is not robust enough to identify these modifications.

Single strand conformational analysis is a similar approach but assesses DNA which has been denatured into single strands and is a more specific technique for identifying mutations. The banding patterns found in both RFLP and SSCP analyses in this study show no obvious deletions or mutations arising from radiation. The fact that a high level of radiation (i.e. 5 Gy) is used certainly questions the appropriateness and suitability of using these techniques to assess damage to DNA in fish tissue cultures. At such high exposures, damage would be expected.

The result based on RT PCR in this study identify cells of rainbow trout gill and skin tissues as having similar level of mitochondrial content and that these tissues show mitochondrial levels considerably higher than that found in spleen. High levels of mitochondria correlate with increased metabolic activity in the cell, replicating their DNA and dividing principally in response to energy needs rather than in synchrony with the cell cycle. These results would concur with this phenomenon. Increased levels of CS found in gill and skin would support this model – tissues with increased energy demand such as skin and gill in fish contain higher mitochondrial numbers. Indeed while the spleen plays a vital role in the generation of blood cells in rainbow trout, the energy required by tissues which are exposed to the environment may expend energy for continuous regeneration and maintenance. Gill and skin also function in oxygen and solute transport into and out of the organism, functions likely to require considerable energy. These results also concur with the citrate synthase levels at the control time point which is an established marker of active mitochondria. Inconsistencies do emerge from these results over time and following irradiation. The highest and lowest levels of mitochondrial content recorded using the RT-PCR data was gill and skin. These values were achieved in tissues following irradiation at 5 Gy at the 48 hour time point and 0.5 Gy at the 2 hour time points respectively. However, the levels of mitochondrial content in gill and skin were, overall, higher than those found in spleen thus supporting evidence found by others.

Mitochondrial content, based on RT-PCR results from gill tissue samples, increase from the low at 2 h control. In gill and skin tissue a distinct ‘U’ shape pattern appears especially the 2 hour time point (skin) and the 96 hour time point (gill) suggesting that lower levels of radiation reduce the levels of mitochondrial content while higher levels are stimulatory. It may suggest that fish skin and gill tissue cultures exposed to

high dose may require increased levels of mitochondria to enter apoptosis. Another possibility could be that mitochondria must be increased in the toxic environment to dilute out significant damage to its genome, in the absence of other protective mechanisms. It is clear from these results that different tissue specific responses exist following direct exposure to radiation – spleen tissues exhibiting a different response to both gill and skin tissues.

In any case, an interesting dose effect pattern may exist where mitochondrial content is inhibited at relatively low radiation doses and stimulated at high doses. While not significant throughout, the pattern observed in the citrate synthase data at the 2 h and 48 h time-points supports the RT-PCR data in this regard. In nuclear DNA, a radiation dose threshold is proposed by some where repair mechanisms intervene if the damage is not significant and apoptosis is activated if the damage is deemed to be too extensive to repair efficiently (31). In the case of mitochondrial DNA, could an opposing mechanism exist whereby there is a mitochondrial termination event at low dose and an amplification of biogenesis following high dose? Certainly one of the main mechanisms open to the mitochondrion for the prevention of transfer of damaged mtDNA is the 'dilution effect' where increased biogenic activity reduces the possibility of defective mtDNA being transferred intra-generation.

Subsequent time-points show decreasing levels of the mtDNA genomes relative to nuclear genomes. While no significant differences exist, the CS activity for skin at the 2 h time-point broadly corresponds to these data, supporting the notion that a similar result is achieved albeit possibly via a different mechanism in skin. Certainly, others have found that melanin, which is abundant in fish skin tissues, modifies the effects of radiation considerably (32). However, given the extensive nature of the significant

differences found in skin tissues at all-time points, the RT PCR data would not support this view. Conversely, while the spleen RT PCR data is less conclusive the clear dose response patterns observed in CS activity at 2 h and 96 h for spleen suggest that the tissue may be sensitive to the effect of radiation.

To conclude, while the results in this study are not conclusive there are considerable similarities in the patterns of response between the RT PCR data and the CS data. This suggests that primers designed in this study for rainbow trout are useful to a certain degree for the monitoring of mitochondrial DNA copy number following exposure to toxic doses of radiation. Further refinement may be useful in this regard. However the approach which focuses on the mitochondrial biogenesis as a marker of toxic stress facilitates the application in *in vitro* technologies and also maintains a close /direct link with the organisms which suffer from exposure to the toxins. The application of real time PCR introduces accuracy, speed and simplicity to the assay process. Using this technology, an assessment of the relative number of mitochondria can be assessed. We show that, similar to those results found in other cell lines and organisms, by increasing toxic exposure, through radiation in this case, positively affects the numbers of mitochondria relative to nuclear. Essentially, stressed tissues produce more mitochondria in a reaction. This facilitates the tissues to overcome the exposure with adequate intact and functioning mitochondria. RT PCR results in this study suggest that tissues in rainbow trout vary in the levels of mitochondria depending on a number of factors. These include tissue type, time post dose and dose intensity. This is supported by earlier work where we saw tissue specific differences following exposure to radiation.

3.6 Acknowledgements.

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CHAPTER 4 THE RELEASE OF BYSTANDER FACTOR(S) FROM TISSUE EXPLANT CULTURES OF RAINBOW TROUT (*ONCHORHYNCHUS MYKISS*) POST EXPOSURE TO γ RADIATION.

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4 Figures, 3 Tables.

Bystander responses in rainbow trout tissue explants.

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4.1 Abstract.

The bystander response has been documented in cell lines and cell cultures derived from aquatic species over the past number of years. However, little work has been undertaken to identify a similar bystander response in tissue explant cultures from fish. In this study, indirect effects of ionising γ radiation on tissue explant cultures of fish were investigated. Tissue explants in culture were exposed to 0.5 Gy and 5.0 Gy γ radiation from a Co^{60} teletherapy unit. A bystander response in *Epithelioma papullosum cyprini* (EPC) cells exposed to γ irradiated tissue conditioned media from rainbow trout explants was investigated and the effects on cell survival quantified by the clonogenic survival assay. Dichlorofluorescein and rhodamine 123 fluorescent dyes were used to identify alterations in reactive oxygen species (ROS) and mitochondrial membrane potential (MMP), respectively. Results indicate a different response for the 3 tissue types investigated. Clonogenic assay results vary from a decrease in cell survival (gill) to no effect (skin) to a stimulatory effect (spleen). Results from fluorescence assays of ROS and MMP show similarities to clonogenic assay results. This study identifies a useful model for further studies relating to the bystander effect in aquatic organisms *in vivo* and *ex* vivo.

4.2 Introduction.

The 'bystander response' is a phenomenon whereby non-irradiated cells exhibit radiation-like responses when in the vicinity of irradiated cells, or exposed to growth medium transferred from irradiated cells. The effect may manifest as a reduction of surviving cells exposed to the conditioned medium resulting from induction of apoptosis, genomic mutation or instability *(1, 2, 3)*. The significance of this effect can only be truly evaluated, in terms of risk assessment to patients or regulation of exposure levels, by investigating the bystander response *in vivo*. Mothersill *et al. (4)* studied the bystander effect *in vivo* in murine bladder epithelium. Studies attempting to address this problem have also exposed *ex vivo* tissue explants of human urothelium to irradiation and transferred the culture media to a stable cell line with a defined response to bystander factors *(5)*. Variations in the level of response were noted depending on a number of factors including genetic background, gender, smoking status and existence of bladder malignancy *(5)*. A previous study by Mothersill *et al. (6)* demonstrated that while irradiated human epithelial cells inhibit clonogenic survival in unirradiated cells, a similar experiment using irradiated human fibroblasts (MSU-1) showed no effect. The existence or amplitude of the bystander response is therefore not ubiquitous in all cell or tissue types. Indeed while many focus on toxic bystander effects, several accounts of stimulatory and adaptive effects are also seen in the historical literature *(7-12)*.

Due to an increased concern about effects of radioactivity on non-human species, several studies have investigated the *in vivo* and *in vitro* responses to direct radiation on mammals, plants and fish. These have been reviewed by Real *et al. (13)* as part of the development of a framework for the assessment of the environmental impact of radiation. It is clear from this that a considerable amount of work has been

carried out fish radiobiology. Most of the work relates to acute exposures of fish to a variety of sources including γ radiation. Real *et al*. *(13)* reviewed 34 studies relating to chronic or low level exposures evenly distributed between freshwater, marine and anadromous species. Most of these relate to reproductive studies which targeted the most sensitive embryonic stages. Of these, data suggest that dose rates of $<4 \times 10^3$ μGy h^{-1} of γ radiation are unlikely to affect survival. Investigators found considerable reduction in the testis size and sperm count in rainbow trout, plaice, guppy, medaka and eelpout when exposed to dose rates of $\leq 1–5 \times 10^3$ μGy h^{-1} of γ radiation. Reduced courtship and immune response were also affected in rainbow trout at these doses. These values appear to be similar in effects to other non-human mammalian species. Knowles *et al*. *(14)* concluded that studies carried out on plaice testes showed similar radio-sensitivity to mammalian testes.

The aquatic environment, and consequently its biota, is more susceptible to radioactive pollution than most other environments. Monitoring of radioactive pollution in fish is routinely carried out to ensure that humans are not exposed to significant levels of radioactivity through the food chain. While nuclear testing has ceased, outputs to the aquatic environments continue from reprocessing plants, nuclear power plants and nuclear powered vessels *(15)*. In these contexts and given that the bystander signal can induce radiation-like damage in non-irradiated cells, defining the response of aquatic biota to radiation exposure is certainly necessary. Studies have already identified bystander responses from fish cell lines such as chinook salmon embryo (CHSE) *(16)* and in hematopoietic tissue cultures of the prawn, *Nephrops norvegicus (17*). Moreover, Dowling *et al. (16)* and Olwell *(18)* carried out clonogenic experiments on the EPC fish cell line exposed to bystander media. However, with these exceptions, little work has focused on the indirect or non-

targeted response effect, which radiation exposure stimulates in aquatic biota. The effects of bystander factors generated from whole fish tissues, has not been adequately investigated to date. Indirect effects of radiation, such as bystander effects, on fish tissue cultures could have implications in areas of ecotoxicology, radiation dosimetry, food safety and regulation/risk evaluation. This study aimed to investigate a potential bystander response from fish tissue cultures of the rainbow trout, *Oncorhynchus mykiss*, *ex vivo*, post exposure to γ radiation. Irradiated tissue conditioned media (ITCM) was generated from trout gill, skin and spleen explants. The effect of this conditioned media on cell survival was investigated, using the clonogenic assay in which the EPC cell line was used as a reporter system. The EPC cell line is an adherent epithelial type cell line isolated from a herpes virus induced hyperblastic lesion on the common carp, *Cyprinus carpio*. It is commonly used, particularly in the diagnosis and isolation of fish viruses. The suitability of the EPC cell line as a reporter culture for radiation studies was confirmed by using irradiated cell conditioned media (ICCM) generated from EPC cultures in a clonogenic survival assay. In an effort to further characterise the effect of ITCM on exposed cells, ROS and MMP load post exposure were also determined.

4.3 Materials and Methods.

Fish

Rainbow trout (*Oncorhynchus mykiss*) of ~200 g average weight were collected at Rafford Trout Hatchery, Athenry, Co. Galway, Ireland (Prop. Francis and Marian Burke). Fish were held in aerated freshwater tanks for ~4 hours. The fish were humanely sacrificed by overdose of anesthetic following guidelines approved by the Dublin Institute of Technology Ethics Committee.

Irradiated Tissue Conditioned Media Generation

Gill, skin and spleen tissue was excised from rainbow trout immediately post sacrifice. Tissue sections were mounted in T25 tissue culture flasks (Sarstedt, UK) containing 5ml MEM (Minimum Essential Medium), (Sigma-Aldrich, UK) supplemented with 10% (v/v) foetal calf serum (Gibco, UK), 2mM L-glutamine (Gibco, UK), 40IU/mL penicillin/streptomycin (Gibco, UK), 30mM Hepes buffer (Gibco, UK) and 1% (w/v) non-essential amino acids (Gibco, UK). Flasks were incubated overnight at 22^{o}C to allow explant attachment. The explants were then exposed to either 0 Gy, 0.5 Gy or 5.0 Gy γ radiation from a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin 6, Ireland) with a dose rate of 1.8Gy/min at a source to flask distance of 80cm. Media (now ITCM) was removed 2 hours post exposure, filter sterilised and stored at -20^{o}C until required for testing using EPC cells.

Clonogenic Assay.

A clonogenic or colony forming assay adapted from the original description by Puck and Marcus *(19)* was used to investigate the effects of ITCM on cell survival. Six hundred EPC cells were seeded into T25 flasks containing 5 ml MEM supplemented as for primary explant cultures and allowed to attach overnight. Medium was then replaced with 5ml ITCM and flasks were incubated at 22^{o}C for 15 days. Cells were stained for 5 minutes with 20% carbol fuschin to aid visualisation. A group of 50 or more cells was considered a colony. Surviving fractions were calculated and compared to those of control flasks.

To confirm the suitability of the EPC cell line as a reporter system for use in bystander studies, T25 flasks containing 5 ml of growth medium were seeded with 7.5 x 10^{5} cells and incubated overnight (~16 hours). Cells were then exposed to either 0 Gy, 0.5 Gy or 5.0 Gy and the media (now ICCM) was harvested 2 hours post exposure, filter sterilised and frozen at –20^{o}C until required for use in the clonogenic

assay. A clonogenic experiment as described above was then prepared using the ICCM.

Reactive Oxygen Species (ROS) Assay

2,7 dichlorofluorescein (DCF) (Molecular Probes, The Netherlands) was employed as a marker for ROS load in EPC cells following exposure to ITCM. Highly fluorescent DCF is generated following the diffusion of 2,7 dichlorofluorescein diacetate (DCFH-DA) into the cell where it undergoes enzymatic hydrolysation producing DCFH (non-fluorescent DCF). ROS rapidly oxidizes DCFH to form the fluorescent DCF *(20)*. Therefore, increased fluorescence correlates to higher levels of ROS. Ninety-six well microtitre plates (Sarstedt, UK) were seeded with 3 x 10^4 EPC cells per well in 200µl of MEM. The plates were incubated for 16 hours at 22^{o}C to confluency. Media was removed and replaced with ITCM and incubated for 6 hours at 22^{o}C. An external negative control of unexposed MEM was included to test for changes in control ITCM samples. Medium was again removed and the cells washed twice with Ca/Mg Buffer (0.1M $CaCl_2$, 0.1M $MgCl_2$ in phosphate buffered saline). DCF (100µl of 5µM) was added to each well, and the plates were incubated for a further 30 minutes at 22^{o}C. The dye was removed by washing 3 times in Ca/Mg buffer before adding 100µl of the buffer to each well. Fluorescence was measured in a Tecan GENios microplate reader (Tecan, USA) employing excitation and emission wavelengths of 488nm and 525 nm respectively.

Mitochondrial Membrane Potential (MMP) Analysis.

MMP was measured using $Rhodamine_{123}$ (Rh123) (Sigma Aldrich, U.K.). Rh123 is a green fluorescent dye, which accumulates in mitochondria in proportion to their membrane potential, indicative of functional mitochondria and healthy cells *(21)*. Briefly, ninety-six well plates were seeded with 3 x 10^4 EPC cells / well in 200µl of

MEM and incubated for 16 hours at 22°C. Media was removed and replaced with ITCM and incubated for a further 6 hours at 22°C. An external negative control of unexposed MEM was included to test for changes in control ITCM samples. Following incubation, the media was removed and the cells were washed twice with Ca/Mg buffer. Rh123 (100 µl of 5µM) was added to each well and the plates incubated for a further 30 minutes at 22°C. Cells were washed a further 3 times using Ca/Mg buffer before adding 100µl of Ca/Mg buffer to each well. Fluorescence was measured in a GENios microplate reader (Tecan, USA) employing excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Statistical Analyses

All statistical analyses were carried out using the Sigma Stat software package (SPSS Inc., U.S.A.). The data presented are mean values +/- standard error for 3 to 5 independent experiments incorporating at least 3 replicates per experiment. Significance was determined using the students *t-test* and differences were considered significant if $p \leq 0.05$.

4.4 Results.

Clonogenic Assays

The effects of direct radiation exposure on the EPC cell line are shown in Figure 4.1. There is no significant effect when cells are irradiated at 0.5 Gy. However, there is a highly significant effect when EPC cells are irradiated at 5 Gy ($P < 0.001$). The surviving fractions of directly irradiated EPC cells are 96% ± 4 and 53% ± 2 respectively. A small but significant ($P = 0.007$) bystander effect was observed in EPC cells post exposure to medium derived from 5.0 Gy irradiated EPC cells. This confirms their suitability for use as a reporter system in this study. The surviving

fractions for EPC cells exposed to ICCM derived from 0.5 Gy and 5.0 Gy exposed cells were 90% and 83% respectively when compared to that of control.

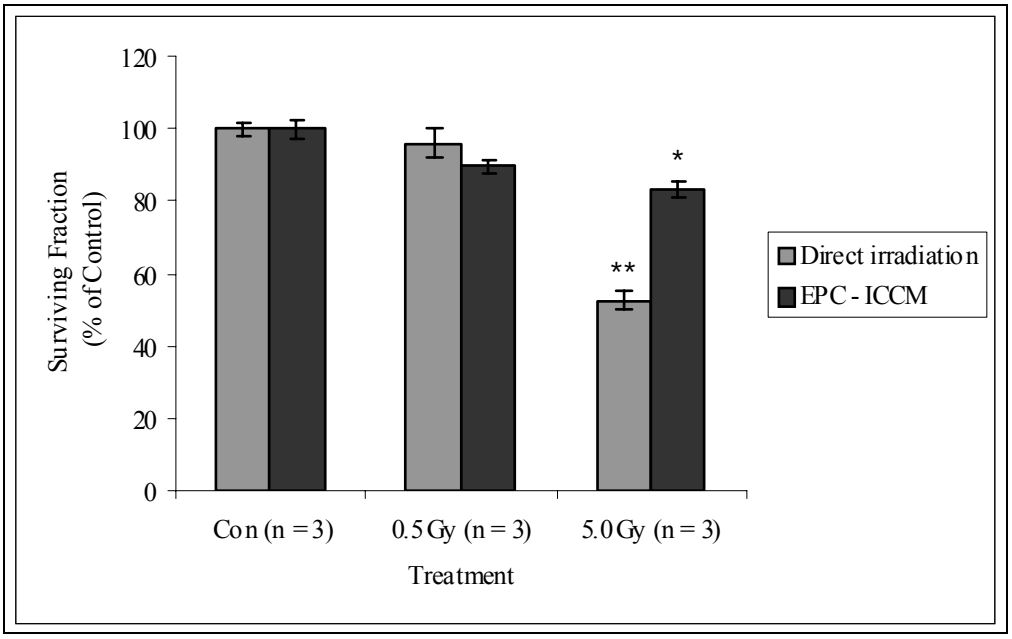

Figure 4.15Surviving fractions of EPC cells following exposure to direct irradiation of 0 Gy, 0.5 Gy and 5.0 Gy and irradiated cell conditioned media from confluent EPC cultures exposed to 0 Gy, 0.5 Gy and 5.0 Gy γ irradiation. Values are the mean of 3 independent experiments and errors are expressed as the standard error of the mean. P-values less than 0.05 and 0.005 are considered significant and are denoted by * and ** respectively.

The clonogenic analysis shown in Figure 4.2 indicates a non-uniform response to ITCM derived from the different rainbow trout tissue types. There is no significant difference in the survival of EPC cells exposed to ITCM derived from rainbow trout skin tissues. In contrast, exposure to 0.5 Gy and 5.0 Gy ITCM from spleen tissues induced a positive growth response in EPC cells. Conversely, the ITCM derived from rainbow trout gill tissues significantly reduced the surviving fraction of EPC cells with increasing dose. The 0.5 Gy ITCM reduced the surviving fraction to 42% and 5.0 Gy ITCM treatment further reduced the surviving fraction to 34%. The plating efficiencies for the clonogenic analysis varied in control ITCM exposed EPC cells indicating that the unirradiated tissue explants had some form of inhibitory effect. Table 4.1 shows the plating efficiency of cells exposed to ITCM from the various tissue types. Considerable differences are evident in the plating efficiencies of EPC cells exposed to ITCM from non-irradiated explants. While control ITCM derived from spleen give a plating efficiency of 36%, both skin and gill control ITCM gives plating efficiencies of 25% and 24% respectively.

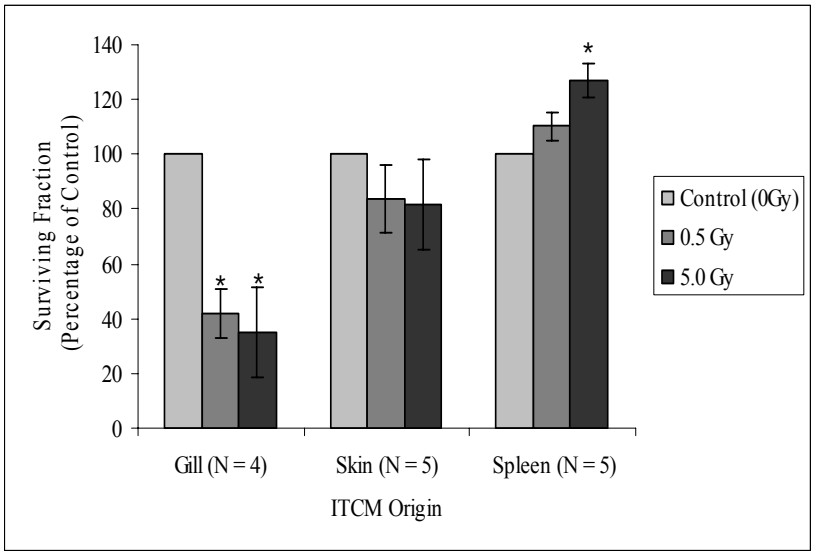

Figure 4.2 Surviving fractions of EPC cells following exposure to ITCM derived from rainbow trout spleen, skin and gill tissue explants. All three tissue types were exposed to 0 Gy, 0.5Gy and 5 Gy γ irradiation. Relative surviving fractions are expressed as the standard error of the mean. A P-value less than 0.05 is considered significant and is denoted by *.

Irradiation Dose (Gy)	Spleen ITCM	Skin ITCM	Gill ITCM
0 Gy	36± 12	25 ± 12	24 ± 10
0.5 Gy	39 ± 14	23± 12	10 ± 6
5.0 Gy	43 ± 14	22 ± 12	8 ± 4

Table 4.1 Plating efficiencies (PE) of EPC cells in clonogenic assays exposed ITCM derived from spleen, skin and gill of rainbow trout.

Reactive Oxygen Species

Figure 4.3 illustrates the differences in ROS levels between EPC cells exposed to control (0 Gy), 0.5 Gy and 5.0 Gy ITCM. Values are expressed as a percentage of ROS levels in EPC cells exposed to control ITCM for each tissue type. ITCM derived from irradiated skin is the only tissue type to have shown an increase in ROS above control level. This increase appears to rise in a dose dependent manner to 117% ($P = 0.021$) and 131% ($P = 0.022$) that of control, in 0.5 Gy and 5.0 Gy respectively. Conversely, ITCM derived from gill tissue exposed to 5.0 Gy produced a significant reduction (89%, $P = 0.039$) of ROS in EPC cells compared to control. There was no significant difference observed in the levels of ROS generation in EPC cells exposed to 0.5 Gy (99%) gill derived ITCM, nor either of the 0.5Gy and 5.0 Gy spleen ITCM treatments (106% and 98% respectively). Table 4.2 is included to show the differences in ROS generation between EPC cells exposed to control ITCM and the external negative control. The level of ROS generation in EPC cells exposed to control ITCM from gill was 179% ($P < 0.005$) that of external negative control. Similarly, the level of ROS generation in EPC cells exposed to control ITCM from skin control was 112% ($P = 0.049$) that of external negative control. Conversely, there was no significant effect on ROS level in EPC cells exposed to control ITCM derived from spleen when compared to external negative control.

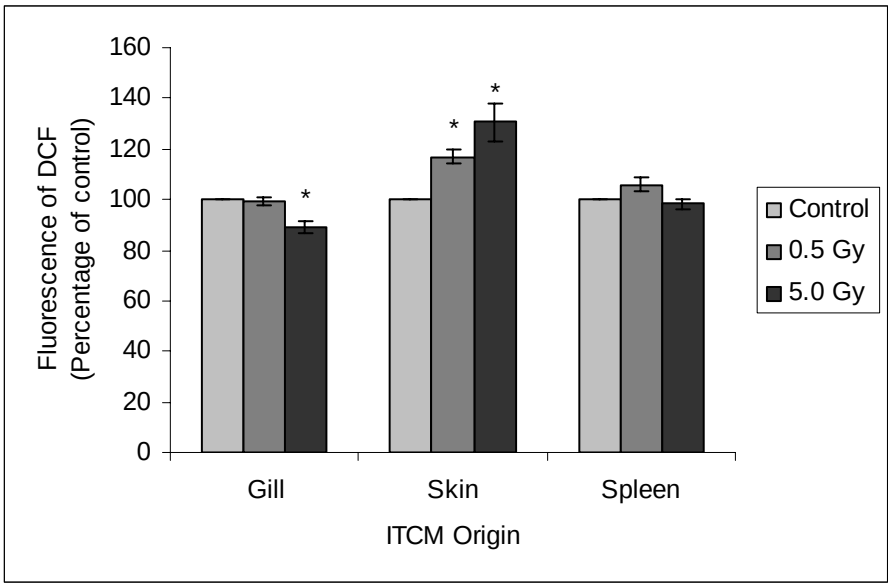

Figure 4.3 Fluorescence of DCF as a percentage of control, in EPC cells exposed to ITCM derived from gill, skin and spleen tissues of rainbow trout, which had been exposed 0 Gy, 0.5 Gy and 5.0 Gy irradiation. Fluorescence intensity of DCF is directly related to ROS. Values are the mean of 3 repeat experiments with 3 replicate values, and errors are expressed as the standard error of the mean. A P-value less than 0.05 is considered significant and is denoted by *.

ITCM Treatment (Gray)	Fluorescence of DCF (Percentage of external control +/-S.E.)		
	Gill ITCM	Skin ITCM	Spleen ITCM
0 Gy	**179 ± 8	*112 ± 5	91 ± 4
0.5 Gy	**177 ± 10	**130 ± 6	97 ± 6
5.0Gy	**159 ± 9	*146 ± 14	*90 ± 4

Table 4.2 Fluorescence of DCF in EPC cells exposed to ITCM from rainbow trout tissues expressed as percentage of external control

* denotes $P \leq 0.05$, ** denotes $P \leq 0.005$

Mitochondrial Membrane Potential

Figure 4.4 illustrates the differences in MMP levels in EPC cells post-exposure to ITCM from each tissue type exposed to 0 Gy, 0.5 Gy and 5.0 Gy. Values are expressed as a percentage of the control. ITCM derived from gill exposed to 0.5 Gy and 5.0 Gy irradiation showed significantly ($P \leq 0.005$) increased MMP in EPC cells, both of which are 116% of the control (0 Gy) ITCM. Conversely MMP levels in EPC cells cultured in ITCM derived from skin exposed to 0.5 Gy and 5.0 Gy were lower than that of the respective control. The 5.0 Gy skin derived ITCM was significantly lower (81%, $P = 0.014$) than the control. MMP levels in EPC cells exposed to spleen derived ITCM showed mixed activity with no change in the ITCM from spleen exposed to 0.5 Gy and a highly significant (183%, $P = 0.002$) increase in MMP levels following exposure to ITCM from spleen irradiated with 5.0 Gy. In Table 4.3, MMP levels are expressed as a percentage of the external negative control to avoid any effects which irradiation may have on the control media. EPC cells exposed to ITCM derived from non-irradiated gill and skin tissues exhibited a significant reduction in fluorescence (60% and 82%, respectively) compared to the external negative control. However, EPC cells exposed to ITCM from non-irradiated spleen showed no significant reduction of MMP levels compared to the external negative control.

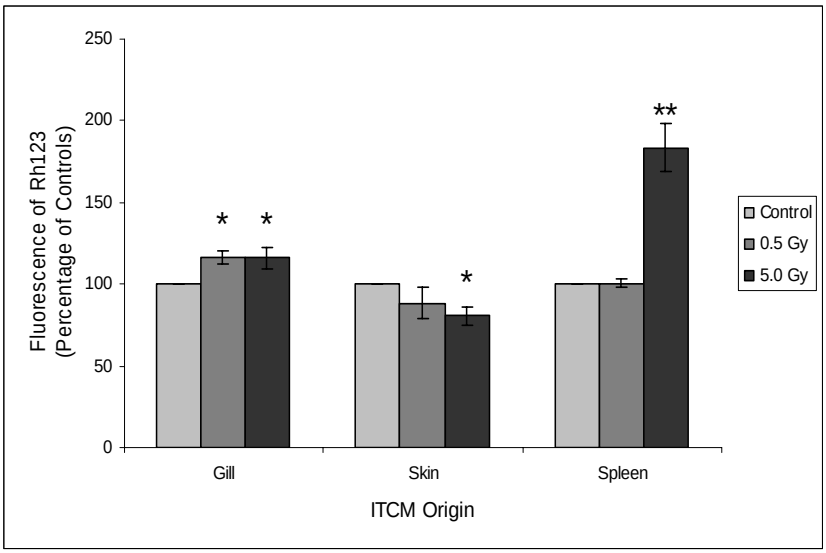

Figure 4.4 Fluorescence of Rh123 as a percentage of control in EPC cells exposed to ITCM derived from gill, skin and spleen tissues of rainbow trout, which has been exposed to 0 Gy, 0.5 Gy and 5.0 Gy γ irradiation. Fluorescence intensity is directly related to MMP. Values are the mean of 3 repeat experiments and errors are expressed as the standard error of the mean. P-values less than 0.05 and less than 0.005 are considered significant and are denoted by * and ** respectively.

ITCM Treatment (Gray)	Fluorescence of Rh123 (Percentage of external control +/-S.E.)		
	Gill ITCM	Skin ITCM	Spleen ITCM
0 Gy	**60 ± 1	*82 ± 4	87 ± 8
0.5 Gy	**70 ± 3	**63 ± 2	87 ± 6
5.0Gy	*70 ± 5	*66 ± 6	**157 ± 8

Table 4.3 Fluorescence of Rh123 in EPC cells exposed to ITCM from rainbow trout tissues expressed as percentage of external control.

* denotes $P \leq 0.05$, ** denotes $P \leq 0.005$

4.5 Discussion.

The ‘bystander response’ is a phenomenon whereby non-irradiated cells exhibit radiation-like damage when in the vicinity of irradiated cells, or exposed to growth medium transferred from irradiated cells. The amplitude or even existence of the bystander response is not ubiquitous in all cells/cell types; studies have however identified bystander responses from fish cell lines including members of the salmonid group to which rainbow trout belong *(16,18)*. In this study, the existence of a bystander response from γ irradiated rainbow trout gill, skin and spleen tissues was investigated *ex vivo*. The suitability of EPC cells as a reporter system for bystander studies was confirmed by exposing EPC cells to irradiated EPC cell conditioned media. The results shown in Figure 4.1 support earlier results obtained by Olwell *(18)* in which a bystander effect was observed. The EPC cells were then incorporated into a clonogenic assay as a reporter system to test media in which fish skin, gill and spleen samples had been exposed to 0 Gy, 0.5 Gy and 5.0 Gy γ radiation.

The results of this study indicate that bystander factors are released from rainbow trout tissues into the culture media post irradiation. Interestingly, each tissue type exhibits a different response. Skin appears to be largely unaffected by radiation with no significant bystander response observed in EPC cells. Average values from the replicate experiments, in which EPC cells were exposed to skin derived ITCM, were suggestive of an inhibitory dose response with increasing radiation. Large error margins between these experiments suggested that this impotency in skin tissues in this study varied in intensity or were dependent on other factors. Mosse *et al. (22)* showed that melanin induces radio-resistance in human cell lines. The high concentration of melanin in fish skin cells *(23)* could confer radio-resistance to this tissue type and may offer one explanation for the absence of an effect in EPC cells

exposed to rainbow trout skin derived ITCM. Conversely, both gill and spleen, which do not contain melanin may be more susceptible to the effects of a bystander factor. Levels of melanin in fish skin vary depending on seasonality, culture conditions and disease status *(24)*. As the fish in this study were collected at different times during the year, a variation in consequent ITCM generation is a possibility. Further studies relating to the effects of melanin levels in skin tissues are recommended.

A bystander response from fish tissues, seen as an 'hormetic' type effect in subsequent clonogenic assays has not been reported to date. Hormesis is commonly associated with growth response, and other manifestations including metabolic effects, longevity, reproductive responses and survival *(8)*. Many workers have reported positive effects, which direct radiation exposure induces in biota *(25-28)*. It is unclear as to how direct radiation stimulates this positive response.

In this study, the stimulatory effect in EPC clones cannot be fully regarded as being hormesis as the stimulation is not effected by direct radiation but indirectly via a signal emitted from spleen tissue exposed to direct radiation. Therefore, the stimulatory effect, identified in this study, may be better described as a radiation induced stimulatory effect (RISE). This is triggered by irradiation but effected via a bystander signal, which, unlike typical bystander responses, confers stimulation rather than inhibition. Indeed, the dose related stimulation may be an important factor when investigating the mechanism of this activity. The nature of the spleen tissue, in terms of structure or function or both obviously has a mechanism whereby a toxicant, such as radiation exposure, can trigger the release of a response signal, which counteracts the initial toxic assault to the extent that a stimulatory growth effect is observed. Moreover this mechanism is most likely dose responsive and not found in the gill or skin tissues.

Several studies have established the link between ionising radiation and the activation of NF-κB, the anti-apoptotic transcription factor associated with many cellular processes including the up-regulation of genes coding for the expression of adhesion molecules *(29, 30)*. These molecules include the intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E selectin *(31, 32)*. Indeed the authors noted increased attachment of spleen tissues following irradiation. Certainly, ITCM enriched with adhesion molecules could alter the response of the clonogenic assay by stimulating EPC cell attachment, thereby increasing the clonogenic survival of EPC cells, as opposed to limiting them by apoptosis and/or necrosis. The association of NF-κB with lymphocytes, - the predominant cell type of the spleen may support this theory.

The inhibitory responses detailed in other studies have pointed towards disruption of the electron transport chain, generation of ROS and initiation of the apoptotic cascade *(33, 34)*. In this study, the generation of ROS (Figure 4.3) in EPC cells treated with ITCM may partially explain the inhibition of clonogenic survival of EPC cells (Table 4.1) shown in this study. However, the levels of ROS measured in the EPC cultures exposed to gill derived ITCM (Table 4.2) are not consistent with the reduction in clonogenic survival of these cells. If ROS were solely responsible for clonogenic inhibition evident in cells exposed to gill derived ITCM, then low survival should also be found in the control samples. These data therefore suggest that an alternative pathway exists which either negates the effects of ROS in controls or inhibits clonogenic survival independent of ROS. Furthermore, this only appears to occur in EPC cells exposed to gill derived ITCM.

The gill tissue, in its role in oxygen exchange in the organism, is mitochondria rich and therefore, has the potential to generate higher levels of ROS than either skin

or spleen when disrupted by toxic assault. The discrepancies in the plating efficiencies between the control samples in this study appear to be ROS dependent and may be related to the level of mitochondrial content or functional energy requirements of the different tissue types. If this is the case, the results in Table 4.2 suggest that any disruption of the tissue, whether it is of a toxic nature or a physical disturbance (*i.e.* removing the tissue from the fish) will generate significant levels of ROS. This phenomenon may have implications in the application of *in vitro* bioassay platforms in environmental toxicology when attempting to validate the *in vivo /in vitro* responses. The effects of ROS on the EPC cultures do, to some degree, support the MMP results (Figure 4.4 and Table 4.3). MMP reduction is typically a result of heightened activity of ATP synthase and a consequent dissipation of the proton gradient across the inner mitochondrial membrane. This heightened activity is likely a response to a higher energy demand by the cells to maintain homeostasis following exposure to ITCM. Cells with low MMP are more likely to produce ROS due to the high activity of the mitochondria whereas the cells with low ATP synthase (and therefore higher MMP) are less likely to produce ROS. Consequently, MMP is lowest in the control gill tissues (Table 4.3) where levels of ROS are highest (Table 2). Conversely, MMP is highest in EPC cells exposed to the spleen 5.0 Gy ITCM where the ROS was found to be lowest.

To conclude, a clonogenic assay was used in this study to identify bystander responses in EPC cells exposed to media in which rainbow trout gill, skin and spleen were irradiated. The clonogenic assay, often referred to as the 'gold standard' assay for investigations into bystander responses *(35-37)* identified interesting tissue specific differences. It is hypothesized that melanin confers some degree of radio-resistance to rainbow trout skin tissue. ROS and MMP activity are commonly used as

a tool for the assessment of cellular compromise in cell culture studies following toxic challenge *(38-40)*. While ROS and MMP show similarities to clonogenic responses following exposure to ITCM identified in this study, further work is required to confirm a mechanistic link between these responses. While MMP levels found in this study are consistent with the clonogenic results, the ROS results were more ambiguous with fewer consistent similarities. This is particularly true of gill tissue explants in which higher ROS levels were found in the control samples than in the treated samples in contrast to the clonogenic result which shows significant inhibition in treated gill samples. This may suggest that other pathways exist in gill tissue, which reduces the inhibitory effects of ROS in cells exposed to gill derived control ITCM. This study highlights the current gap in knowledge on the effects of radiation in aquatic species and the lack of validated techniques required for fish-based tissue culture systems.

4.6 Acknowledgements.

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CHAPTER 5. THE APPLICATION OF IMAGE ANALYSIS IN ADVANCING THE CLONOGENIC ASSAY FOR POLLUTION STUDIES USING RADIATION AS A MODEL TOXICANT.

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6 Figures, 1 Table.

Application of image analysis in clonogenic assays.

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5.1 Abstract.

O'Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., Lyng, F.M., McClean, B., Seymour, C. and Murphy, J.E.J. The Application of Image Analysis in Advancing the Clonogenic Assay. *Rad. Res.*

The clonogenic assay has been used in effectively its original form since the 1950's, as the preferred method to determine the effects of exposure to toxins, and particularly radiation, *in vitro*. One of the main criticisms of the assay is its susceptibility to subjectivity regarding what constitutes a colony of ≥50 cells. Since thousands of colonies need to be counted in a typical clonogenic assay, objectivity can often be compromised by the laborious nature of the task. Here, we examine the application of image analysis techniques in the clonogenic assay focusing on accurate and consistent colony counting and colony and cell size analysis. Results confirmed the accuracy and sensitivity of the clonogenic assay for use with the EPC cell line in radiation studies. They also identified dynamic changes which are occurring at colony and cellular levels within treated cell culture populations. The application of image analysis to clonogenic assays is not only expeditious but facilitates the simple and objective collection of data relating to cell culture population dynamics. This adds value to clonogenic data and may enhance our understanding of the complex nature of toxic exposure in *in vitro* biological models.

5.2 Introduction.

The clonogenic assay has long been used as the "gold standard" for the identification of effects of toxicant exposure on cell lines and in particular for radiation studies in biological systems (1 - 4). In order to ensure statistically relevant and accurate results, many flasks containing several thousand colonies are typically counted. This work is laborious, repetitive and time-consuming often resulting in loss of objectivity and a consequent deterioration in the quality and reproducibility of results (5 – 9). Achieving objectivity throughout the results is the single greatest challenge of the clonogenic process (6).

Many researchers realize the usefulness of image analysis and colony counting software technology in clonogenic counts (10-12). This technology has proven to be accurate, consistent and expeditious. Moreover, assuming that all of the flasks are analysed under the same conditions, the technology is above all objective. While the technology being applied has improved the clonogenic assay in terms of objectivity, the basis of the clonogenic assay remains unchanged (1). However, with the advances in image capture and analysis technology, additional useful data may be generated from the typical clonogenic flask. For example, the dynamics of the clonogenic population can be assessed including the structure, size and accurate extent of the population. Typical colonies comprising 50 cells can be accurately and objectively identified and colonies which have grown larger than the median colony size may be considered. Previously, such differences were often attributed to contamination or two or more cells being seeded together as a result of clumping in the initial inoculum. However, such shifts in size distribution of the clonogenic population may be an indication of sub-acute and/or molecular responses to toxins. In an effort to characterize colonies of different size, a PCR confirmation step can be introduced. As

colony size and formation is a parameter often used to identify cell type, the inclusion of PCR analysis can determine if colonies of disparate size comprise cells of the same origin or not.

While many studies give details of replicate clonogenic counts, few have described the growth dynamics of these colonies, the accurate identification of '50 cell colonies', or the effects on cell size. We hypothesized that the nature of the colony and its size relative to its sister colonies provides useful additional information in the clonogenic process. In this study we examined a number of techniques based on image analysis and PCR, and assessed their application in the context of clonogenic assays in radiation science. We focused on direct exposure of *Epithelioma papullosum cyprini* (EPC) cells to Cobalt^{60} γ radiation. EPC cells are an adherent epithelial-type cell line isolated from a herpes virus induced hyperblastic lesion on the common carp, *Cyprinus carpio*. We also investigate the indirect exposure of EPC cells to γ radiation using Irradiated Cell Conditioned Media (ICCM) from exposed EPC cells.

Image analysis may be utilized to add value to the standard clonogenic assay data (i.e. plating efficiency and survival fraction) through accurate and consistent identification of the '50 cell' colony and the calculation of colony and cell size differences following exposure. A number of possible indicators of the effects of radiation on a cell line routinely used in clonogenic assay experiments are investigated. A PCR characterization step is included to confirm the origin of divergent colonies in terms of size so as to dismiss the possibility that contamination may be responsible. These techniques and the merit of the data produced are discussed.

5.3 Materials and Methods.

Clonogenic assay of EPC cells exposed to direct radiation.

A clonogenic assay adapted from the description by Puck and Marcus (1) was used to investigate the effects of both direct and indirect irradiation. Eight hundred EPC cells were seeded in T25 flasks in triplicate, each containing 5 ml Minimum Essential Medium (MEM) (Sigma-Aldrich), supplemented with 10% (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 40 IU/ml penicillin/streptomycin (Gibco), 30 mM Hepes buffer (Gibco), and 1% (w/v) non-essential amino acids (Gibco). After overnight attachment flasks were exposed to either 0 Gy, 0.5 Gy or 5.0 Gy γ radiation from a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin 6, Ireland) with a dose rate of 1.8 Gy/min at a source-to-flask distance of 80 cm. The growth medium was replaced with 5 ml of fresh medium following radiation and the flasks were incubated at 22^{o}C for 15 days. Resulting colonies were fixed with neutral buffered formalin (10% v/v formalin in PBS) and stained for 5 minutes with 20% carbol fuschin.

Clonogenic assay of EPC Cells Exposed to Irradiated Cell Conditioned Medium.

T75 flasks (Sarstedt) containing 30ml of MEM growth media were seeded with 7.5 x 10^5 cells and incubated overnight at 22^{o}C. Cells were then exposed to either 0 Gy, 0.5 Gy or 5.0 Gy as above. The media (now ICCM) was harvested 2 hours post exposure, filtered through 0.22 μm pore size porosity filters, and stored at –20^{o}C until required. Eight hundred EPC cells were seeded into T25 flasks in triplicate, containing 5 ml MEM and allowed to attach overnight. Medium was then replaced with 5 ml ICCM and flasks were incubated at 22^{o}C for 15 days. Colonies were fixed and stained as described above.

Image Analysis

An image of each flask was acquired using the ChemiDoc XRS image analysis system (BioRad, Italy). These images were then analysed using ImageJ software

(http://rsb.info.nih.gov/ij/) employing the analytical option 'analyse particle function' where colony number and colony area were measured. The data generated gives the area of each colony in pixels. This pixel value was calibrated by measuring a 60mm x 25 mm coverslip (VWR International) which was placed beneath each flask being analysed. This facilitated the calibration of each flask individually. In a concurrent experiment, sister flasks were prepared as in the clonogenic experiment above. Following the appropriate treatments and incubation, the cells were fixed in neutral buffered formalin and stained with a 0.3% (v/v) solution of toluidine blue (Agar Scientific, Essex, UK). Slides were prepared from the flasks by removing the upper section of the flask and mounting a coverslip using glycerol gel (Dako, CA, USA). Flasks containing EPC cells exposed to either direct radiation or ICCM media were then analysed for cell size differences using a compound microscope (Nikon, Japan) mounted with a digital camera (SPOT). Randomly selected colonies were digitally captured and also analysed using the ImageJ software.

Colony surface area and the surface area of a random selection of individual cells comprising the colony were measured. A cell count of each colony chosen was carried out. This data was used to generate a standard curve with cell number against colony size allowing prediction of the number of cells in any colony for which surface area data existed. This also facilitated the accurate identification of colonies comprising 50 cells or more which is the central tenet of the clonogenic assay. The colony size data from the original clonogenic assay could then be modified by removing any colony data points which contained 50 cells or less. Surviving fractions were calculated and compared to those of control flasks.

Molecular Confirmation Analysis

The primer sets C3 (F1 and R1) and RT10 (F1 and R1) were designed based on the mitochondrial genome of the carp, (*Cyprinus carpio)* and the rainbow trout, (*Oncorhynchus mykiss*) respectively (Table 1). To confirm the origin of morphologically different colonies, samples from individual colonies were placed directly in a micro tube containing 1x Readymix *Taq* (Sigma-Aldrich), and 5μM primers. The PCR reaction conditions were 95^{o}C for 2 minutes then 30 cycles of 94^{o}C for 40 seconds, 56^{o}C for 2 minutes and 72^{o}C for 3 minutes. Reactions were carried out in a Peltier Thermal Cycler, PTC-225 (MJ Research, Massachusetts, USA). PCR products were separated on an agarose gel and visualized using the ChemiDoc system (BioRad, Italy).

Primer		Sequence Origin	Prod. Size	Sequence
Carp C3	F	Carp mitochondria (Accession No. NC_001606)	353bp	acatagccgatcaacgaacc
Carp C3	R			ctgccacccagtatgtcctt
RT 10	F	Rainbow trout mitochondria (Accession No. NC_001717)	800bp	cacagcaggccacctcctaattc
RT 10	R			agtgtcagtatcaggcggcagc

Table 5.1 Details of primers used for confirming origin of cell lines used in this study.

5.4 Results.

Clonogenic assays

A typical captured image of a clonogenic flask is shown in Figure 1. As with most clonogenic flasks, a broad range of colony sizes are evident.

Colony size measurements of control EPC clonogenic flasks were correlated with cell number in Figure 2. The resulting regression equation (correlation coefficient R^2 = 0.7814) was y = 86.953x + 2948.1 indicating that the area of a 50 cell colony is 7295 μm^2 (Figure 2). In these controls, colonies ranged in size from 5300 μm^2 to 39200 μm^2. In colonies comprising 50 – 90 cells there is a strong correlation with size however above this number, this correlation is increasingly erratic.

Figure 3 shows the typical information provided using a normal clonogenic study. EPC cells directly exposed to 0.5 Gy and 5.0 Gy radiation exhibited significant reductions in surviving fractions (82% and 61% respectively). EPC cells exposed to ICCM derived from cells exposed to 5 Gy also exhibited a significant reduction (88%), when compared to that of controls (Figure 3).

Colony Image Analyses

The size of each colony on each clonogenic flask was measured. The average colony sizes on both directly irradiated cells and those exposed to ICCM are shown in Figure 4. Colonies exposed to direct radiation are approximately twice the size of corresponding colonies exposed to ICCM. This is also the case with sham ICCM. There is also a reduction in colony size in cells exposed to direct radiation which was found to be highly significant in the 5 Gy exposure. Conversely, there is a general increase in colony size in EPC cells exposed to ICCM from 0.042 mm^2 in controls to 0.046 mm^2 and 0.045 mm^2 in. 0.5 Gy and 5.0 Gy ICCM respectively. Only 0.5 Gy ICCM is significantly greater in size.

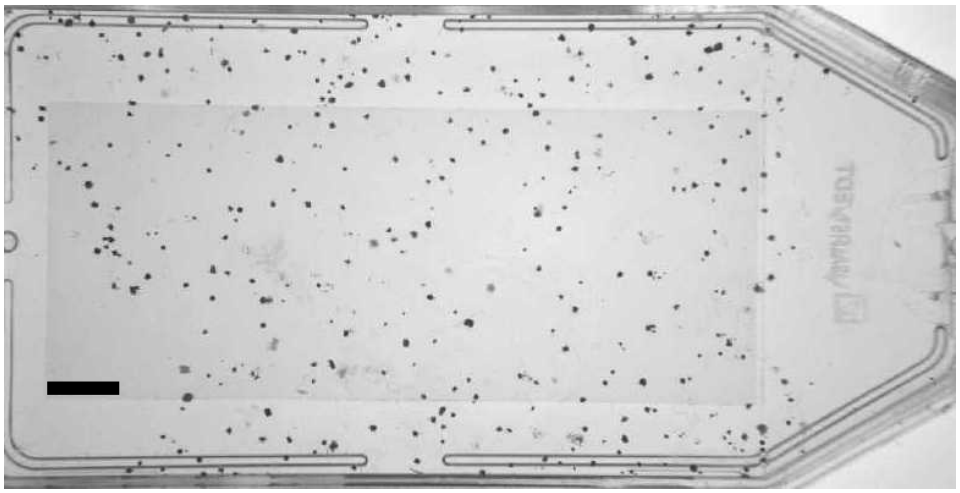

Figure 5.19 A typical clonogenic flask containing EPC colonies following incubation at 22^{o}C for 15 days. Colonies are stained with carbol fuschin. Bar = 6mm

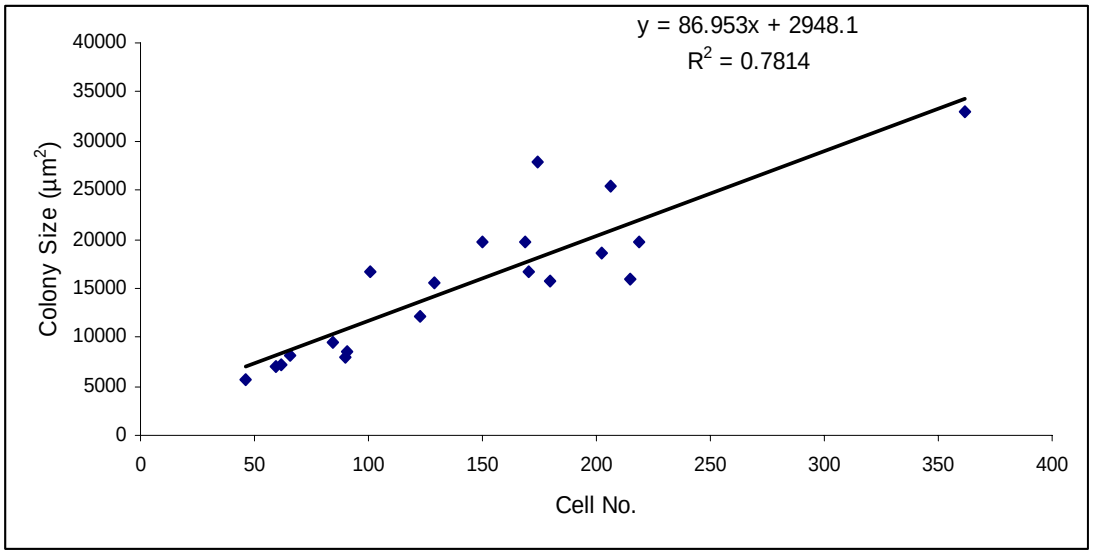

Figure 5.20The relationship between colony size and cell number for EPC cells in control samples. The correlation is represented by the equation y = 86.953x + 2948.1. The R2 is 0.7814.

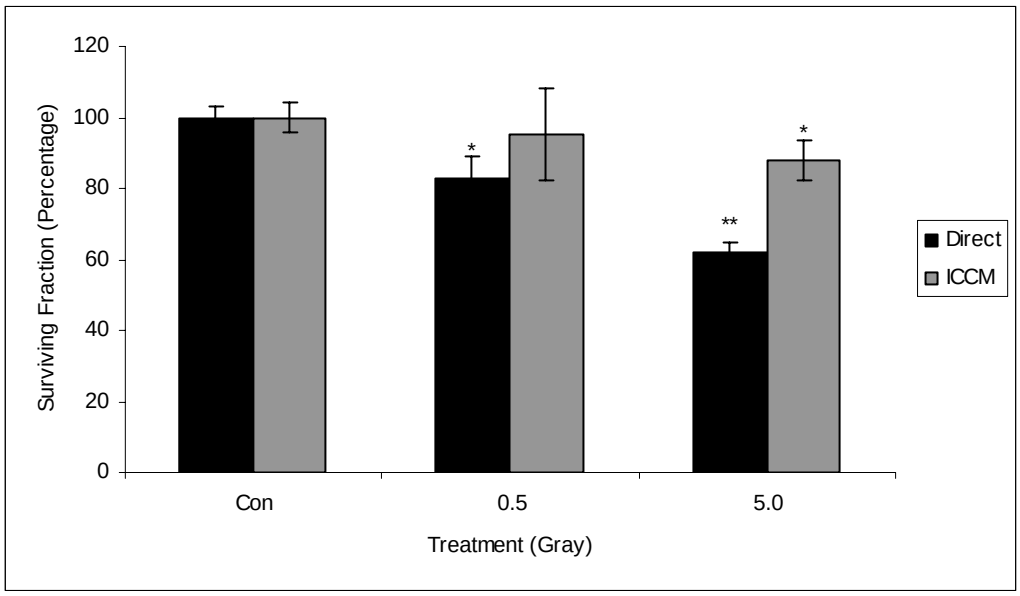

Figure 5.3 Effects of direct radiation and ICCM exposure on EPC cells determined by clonogenic survival analysis. Values are the mean of 3 independent experiments and errors are expressed as the standard error of the mean. P-values less than 0.05 are considered significant and are denoted by *.

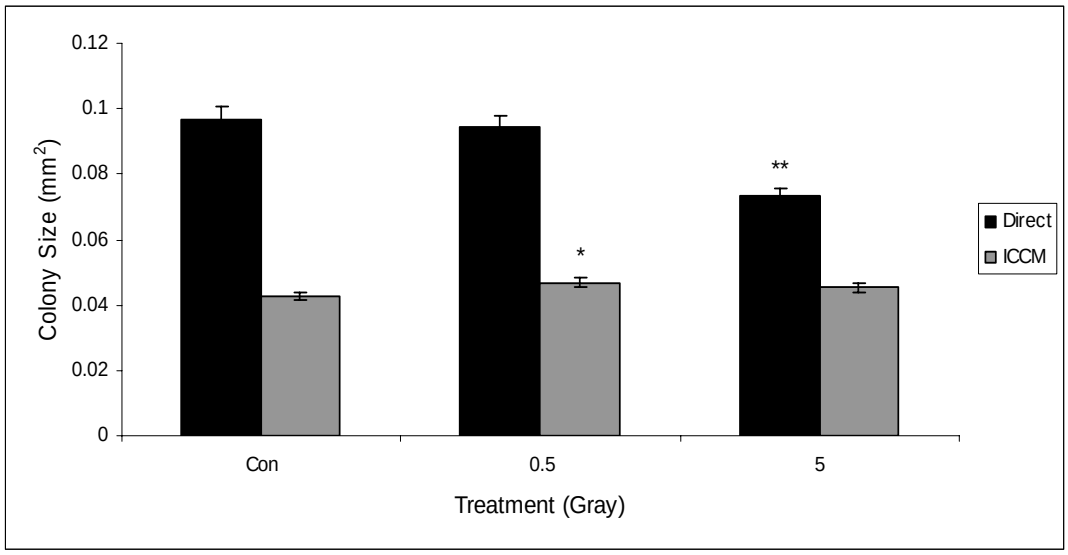

Figure 5.4 The average colony size of EPC cells exposed to direct radiation and ICCM. Colonies were captured digitally using the ChemiDoc system (BioRad) and measured using ImageJ software. Values are the mean of 3 independent experiments and errors are expressed as the standard error of the mean. P-values less than 0.05 and 0.005 are considered significant and are denoted by * and ** respectively.

Cell Size Image Analysis

The size of cells comprising colonies exposed to both direct radiation and ICCM were measured. The data shows that there is a significant increase in cell size in EPC cells directly exposed to 5.0 Gy radiation (Figure 5). Conversely, there is a significant reduction in cell size of EPC cells exposed to ICCM from 5.0 Gy γ-irradiated EPC cells ($P = 0.029$) but not 0.5 Gy which shows no change from control levels.

PCR Analysis

The gel shown in Figure 6 confirmed that the DNA taken from both large and small colonies is of carp origin. Bands appearing in lanes 2 and 4 are positive controls for carp and rainbow trout mitochondrial DNA respectively. Samples taken from both large colonies (lanes 5 – 7) and small colonies (lanes 8 – 10) only amplify the 353 bp product associated with carp and not the 800 bp product associated with rainbow trout.

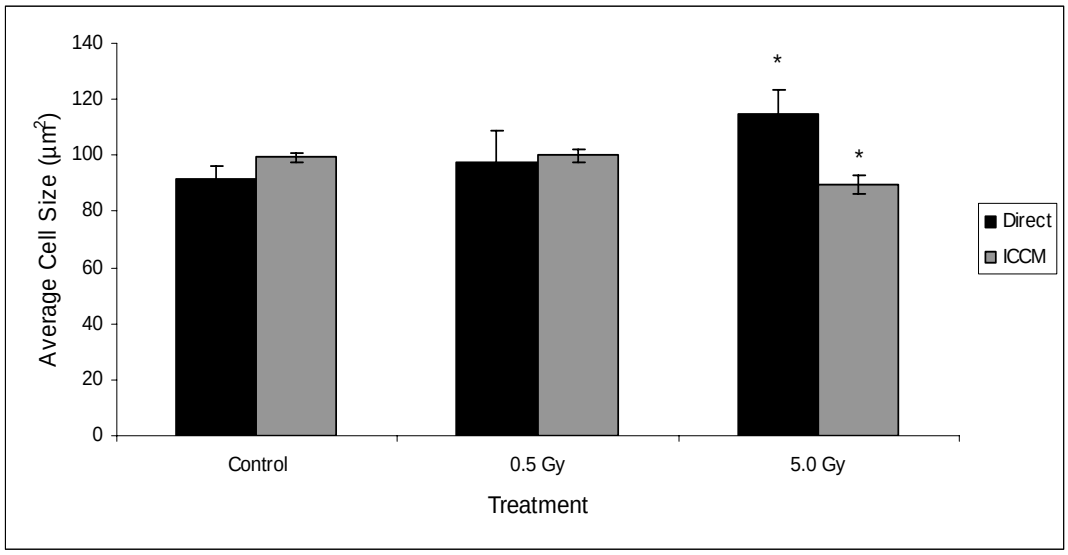

Figure 5.5 The average cell size of EPC cells following exposure to direct radiation and ICCM. Colonies were captured digitally using the ChemiDoc system and measured using ImageJ software. Values are the mean of 10 cell measurements chosen randomly along transects of 5 colonies from each dose regime in triplicate. Each experiment was carried out independently in triplicate and errors are expressed as the standard error of the mean. P-values less than 0.05 are considered significant and are denoted by *.

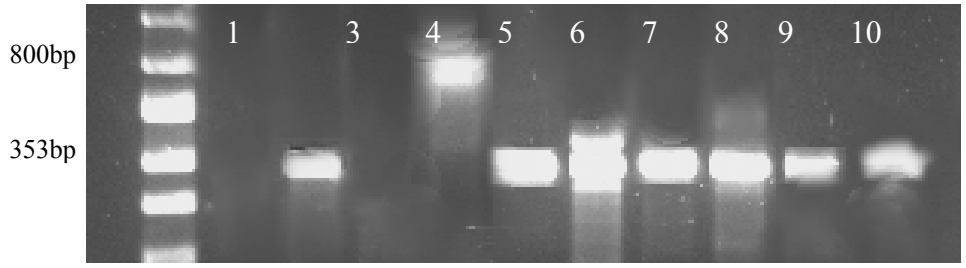

Figure 5.6 PCR analysis of colonies randomly selected from test culture flasks. The sample gel pictured shows products amplified using the Carp C3 primer and samples taken from both large colonies (lanes 6 - 8) and small colonies (lanes 9 - 11) found in clonogenic flasks. Lanes 1 – 2 are negative and positive controls for Carp C3 primer pair respectively and lanes 3 – 4 are negative and positive controls for RT 10 primer pair respectively.

5.5 Discussion.

The clonogenic assay is an *in vitro* test system which examines the ability of a single cell to divide successfully over several generations to form a colony of identical clones. It has been used extensively and to great effect by toxicologists and radiation biologists for several decades to determine cytotoxic effects of test treatments. Indeed, some have suggested that it is the 'gold standard' assay for the identification of toxic assault or for identifying bystander effects in biological systems (2 - 4). However, while few if any can dispute the fact that the clonogenic assay is the benchmark assay system for relative toxicity measurements, some limitations still persist. The manual counting of colonies is tedious, lengthy and often suffers from user subjectivity. Also, contamination can occur especially in those cell lines which require several weeks to form colonies. Over the years, little has changed in the clonogenic assay protocol despite significant scientific advances particularly in areas associated with image capture and analysis technologies. In this study, the application of image capture technologies of clonogenic flasks and subsequent analysis using the associated software has been examined. In an effort to confirm derivation of the atypical colonies formed, a PCR-based identity confirmation step was included.

The image of a clonogenic flask in Figure 1 shows how emergent colonies in a typical assay may vary in appearance and size. Indeed, from the early stages in clonogenic assay development, Puck and Marcus introduced the threshold colony size of 50 cells to eliminate those colonies which undergo several divisions prior to collapse following lethal dose exposure (1). However, as Biston *et al*. (6) pointed out, surviving cells proliferate with diverse doubling times leading to a large range in colony sizes. Furthermore, the clonogenic assay may not be suitable for all cell lines in the way that it was for the cell line for which it was initially developed.

It is interesting to note that the estimated '50 cell' colony using the EPC cell line and based on the linear regression study carried out here covers an area of approximately 0.007mm^2. This is scarcely visible to the naked eye when stained, therefore relying on an objective eye to differentiate at this level would be challenging. The use of image analysis to calibrate cell number against colony size is admittedly time consuming but while once-off measurements may be adequate in cases where the exact same conditions exist, this approach may be too presumptuous for general use in cell culture. The benefits of calibration leave no room for miscalculation or misinterpretation in an assay which is prone to such vagaries and surviving fractions may be calculated accurately and objectively.

The suitability of the EPC cell line as a reporter culture for radiation studies was confirmed by using irradiated cell conditioned medium (ICCM) generated from EPC cultures in a clonogenic survival assay in our previous studies (13). While slight deviations exist in the plating efficiencies, the original result is confirmed. Such differences may be attributable to media batch differences or growing conditions, however some may be associated with the precision afforded by image analysis.

Another interesting feature is the range of colony sizes within this clonogenic study. Indeed several orders of magnitude of difference exist in the control samples from the direct exposed cells. The substantial difference in colony size between directly irradiated cells and those exposed to ICCM is clearly associated with the addition or process of transferring media. While nutrient limitation is a possibility, this is unlikely as the media is fresh prior to irradiation and removed 2 hours post radiation. The only other possibility is that the sterilization through a 0.22 μm filter may have some form of inhibitory or deleterious effect on this media.

We surmise that variations in colony size can be explained by sub-lethal effects brought on by toxic exposures. In the directly exposed cultures in this study, the overall trend in colony size is downward thus correlating with the surviving fraction calculations. It is clear from these results that exposure to radiation does alter the colony size in EPC cells, however calculating the surviving fraction appears to be a more sensitive end-point than assessing distortion of the population structure based on colony size.

Many studies have identified a decrease in colony size (10, 14): Spadinger *et al*. identified a dose-dependent response with a mammalian cell line characterized by fluctuations in the 0 – 1.5 Gy dose ranges citing inducible repair mechanisms as a possible mechanism to explain this phenomenon.

While surviving fractions also decrease with increasing dose-exposed ICCM, there is an increase in colony size suggesting that other mechanisms are at work. Direct radiation inhibits colony size but exposing cells to media from irradiated cells promotes colony size. It appears that radiation exposure may release a component or factor from EPC cells into the culture media which subsequently can be utilized by non-irradiated cells to generate fewer but larger colonies and that this appears to be dose related. This might suggest that cells exposed to ICCM are attracted to each other. These amalgamate and create fewer larger colonies, when activated by factors which are released into the growth media, in a survival mechanism.

What kind of factor could be responsible for such an effect? Earlier work by our group identified stimulatory effects in EPC cells cultured in tissue conditioned media (13). It was proposed that this effect may be associated with the release of adhesion molecules such as VCAM, ICAM and e-selectins. Could this also be the case here? Certainly, cells have some form of inter-communication mechanisms. Many cell

types are density dependent requiring a threshold concentration in order to survive in culture.

Cell size also varies with dose and exposure type. Cells in colonies exposed to direct radiation increased with increasing dose although this was only significant at 5.0 Gy. Conversely, cells in colonies exposed to ICCM significantly decreased in size when exposed to 5.0 Gy ICCM.

This could be similar to ecological principles associated with competition and may not be reaction to dose or exposure. Essentially larger colonies tend to have smaller cells due to competition in the cell 'bundle' while smaller colonies tend to have larger cells as there is less competition for space. Indeed, we can argue that as colonies become smaller with increasing direct radiation, their constituent cells become larger. Conversely, as colonies become larger with increasing ICCM exposure, their constituent cells become smaller. As colonies directly exposed to radiation are approximately twice the size of ICCM exposed colonies, we therefore have to presume that there is another factor which is responsible for the cell size effects identified in this study.

Contamination of the clonogenic flasks is a possibility especially in busy labs where many cell lines are cultured and several users share the same facilities. This study incorporated a PCR characterization step, which set out to confirm the authenticity of disparate colonies that formed within the same flasks. While it is impossible to say that no other contaminating factor is responsible for large or small colonies, the inclusion of the PCR step confirms beyond reasonable doubt that all colonies develop from the original inoculum. Figure 6 confirms that samples taken from both large and small colonies do originate from carp and are therefore, likely to be EPC-derived colonies.

To conclude, the application of image analysis in the clonogenic assay procedure can identify interesting aspects to the growth dynamics in emergent clonogenic populations. The approach is fast, accurate and objective for the generation of plating efficiencies and surviving fractions. Generally however, the traditional clonogenic approach of calculating the surviving fractions appears to be a more sensitive endpoint than colony size or indeed cell size.

This data suggests that alteration in colony size is a common phenomenon associated with EPC cells but size can be modified by toxic exposure. In EPC cells, colony size can be used as an endpoint of γ radiation exposure. However, low dose effects may suffer from lack of sensitivity relative to the traditional clonogenic endpoints.

Direct radiation inhibits colony size formation, but the media from directly irradiated cells promotes colony formation in a dose dependent fashion. The increase in cell size in EPC cells exposed to direct radiation and the decrease in cell size exposed to ICCM are partly explained by competition for space.

We support other authors in their calls for the introduction of image analysis applications as routine practice in the clonogenic assay. Furthermore, while the inclusion/collection of other parameters such as colony size and cell size data may not appear to be any more sensitive than traditional clonogenic measurements, their introduction provides added value and indeed may be more appropriate for other cells lines, dose exposures or experiment designs.

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CHAPTER 6 GENE EXPRESSION AND ENZYME ACTIVITY OF MITOCHONDRIAL PROTEINS IN IRRADIATED RAINBOW TROUT (ONCORHYNCHUS MYKISS, WALBAUM) TISSUES *IN VITRO*.

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O'Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., Lyng, F.M., McClean, B., Talbot, A and Murphy, J.E.J. Gene expression and enzyme activity of mitochondrial proteins in irradiated rainbow trout (*Oncorhynchus mykiss,* Walbaum*)* tissues *in vitro*.

6.1 Abstract.

In recent years ethical, legislative and economic pressures have created a renewed interest in the development of alternatives to *in vivo* whole animal experimentation. *In vitro* studies, particularly those using cell cultures, have been used increasingly as tools to assess the degree of toxicity associated with or present in particular environments. However, it has been noted that while cell cultures are useful to give relative toxicity values, genotypic and phenotypic integrity may be compromised in the continuous artificial environment they inhabit. Equally, cell cultures lack the complexity of functional organs and thus do not truly represent the effects which toxins exert on organ and organism functionality. In this study, *ex vivo* tissue culture of rainbow trout gill, skin, and spleen samples were analysed for variation of expression in genes associated with oxidative phosphorylation following exposure to ionising radiation (IR).

Significant IR-induced changes in gene expression and enzyme activity associated with the mitochondrial oxidative phosphorylation process were identified. The tissues examined in this study demonstrated an exposure threshold at which radiation dose stimulates an alteration in the regulatory activity of mitochondrial associated genes. Spleen tissues exposed to low levels of IR (0.1 Gy) appeared most sensitive whereas skin tissues proved least sensitive reacting only to higher doses (>1 Gy).

We propose this investigative approach as an innovative alternative to *in vivo* studies as it identifies toxic exposure *in vitro* and could significantly reduce the number of live animal toxicity tests required.

6.2 Introduction.

Various organisms and techniques have been used to monitor toxic inputs to the environment (1–3). The aquatic environment has been the subject of many studies relating to pollution by either natural or anthropogenic sources. These include toxins of a chemical, biological and/or physical nature (4). While many of these inputs are easily recognisable shortly after they enter watercourses, many go unnoticed for considerable periods leading to chronic damage over the medium to long term.

The conventional approach to investigating the effects of toxins in the environment is to expose a 'battery' of organisms to a specific toxin / test substance. These experiments are designed to replicate, as much as possible, the theoretical interaction of test substances within a common trophic structure by including organisms from different genus, families and phyla associated with the particular environment where these substances are found. Based on the survival/fatality data generated from these test organisms lethal concentration values for the test substance are calculated (5, 6). In the aquatic environment, organisms typically include members of the micro and macroalgae, bacteria, protozoa, invertebrate and vertebrate families. While this approach is a good indicator of lethal dose concentrations of toxicants in a water body, significant shortcomings exist. Thus, many millions of fish are sacrificed annually in laboratory-based experiments raising moral, ethical, economic and indeed scientific questions relating to this practice.

As a consequence of these pressures, the use of *in vitro* based techniques is becoming increasingly popular. Many studies have centered on the development of *in vitro* based assay techniques to identify toxic exposure. These frequently involve the use of *in vitro* based cell cultures often associated with representatives from the trophic structures of conventional toxicity tests (7).

While the use of cell lines holds several advantages due to their non-reliance on live animal experiments and their usefulness in facilitating molecular and spectrophotometric based techniques including cloning, flow cytometry and growth studies, there are some misgivings associated with their use in toxicity studies. These include concerns relating to the loss of physiological traits associated with the organism which it aims to represent. This is due to the artificial environment in which cell lines are isolated, maintained and reproduced *in vitro* (8). To avoid this problem, some have suggested using primary cultures from specific organisms (9, 10). While this will not completely remove the need for the sacrifice of animals, the nature of primary *in vitro* tissue culture means that many experiments can be carried out using sections from the same organs, thereby reducing the number of animals used in the experimentation. Furthermore, the genotypic and phenotypic traits of the functioning organ more closely represent the living organism.

An increasing emphasis has been placed on gene expression to identify the effect of toxins on biological systems (11-13). The alteration of gene expression following biological, chemical and physical toxic exposures has been identified in several studies in humans, rodents, mice, fish, and invertebrates and arguably represents a more sensitive endpoint than the more classical endpoints in the field at present (14-17).

Gene expression arrays have been used to identify toxic or stress related responses in aquatic species such as Atlantic salmon and rainbow trout (18, 19). These have mainly concentrated on genes associated with the nuclear genome. Surprisingly, few studies have examined the usefulness of gene expression to identify toxic effects associated with the mitochondrion yet the mitochondrion is central to several vital cellular processes, including energy production by oxidative phosphorylation

(OXPHOS), calcium homeostasis and apoptosis. It has its own genome and is able to replicate, transcribe and translate its own DNA independently of the nuclear DNA. However, mitochondrial and cellular functions are interdependent and considerable cross-talk occurs (20). For example, the mitochondrial genome has the translational capabilities for 13 of the 87 proteins necessary for the OXPHOS process in the cell (21). The remaining proteins associated with the mitochondria are derived from nuclear encoded genes.

The concentration of mitochondria in any particular cell relates to the energy requirement and function of the organ from which the cells are derived (22). Organs with high levels of mitochondria include gills of fish where much of the oxygen is processed and the skin across which oxygen diffuses. Functional mitochondria in these tissues are vital to the survival of the organism: any influence of a toxicant on OXPHOS in the mitochondrion is subsequently reflected in the health of that organism. Given their importance, their easy isolation and maintenance and their independent DNA genome, this study has targeted the mitochondrion as a source of biomarkers for toxic stress.

In this study, we have chosen to examine a middle ground. Rather than eliminating the use of live animals which may compromise results to an unacceptable level, we have aimed to significantly reduce the numbers of live animals used by combining *in vitro* based tissue culture techniques with a gene expression study focusing on the functioning of the mitochondrion. The activity of a number of genes encoded by and specific to this function were included. We also included a key enzyme which is encoded by the nuclear genome but specific to the mitochondrion. The nuclear encoded, nuclear specific gene, beta-actin gene (β-actin) was used as a reference 'housekeeper' (23 - 25). Enzyme kinetic analysis was carried out in an effort to

quantify the phenotypic (and functional) impact, if any, of any changes in gene expression.

6.3 Materials and Methods.

Fish

Rainbow trout of ≅ 200 g average weight were collected from a commercial fish farm in Ireland. Fish were held in aerated freshwater for ≅ 4 h before sacrificing with an overdose of anesthetic following guidelines approved by the Dublin Institute of Technology Ethics Committee.

Tissue Explant Preparation and Irradiation

Gill, skin and spleen tissue explants were excised from rainbow trout immediately after death and transferred to T-25 tissue culture flasks (Sarstedt) containing 5 ml MEM (Minimum Essential Medium) (Sigma-Aldrich) supplemented with 10% (v/v) foetal calf serum (Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), 40 IU/ml penicillin/streptomycin (Gibco-BRL), 30 mM Hepes buffer (Gibco-BRL), and 1% (v/v) non-essential amino acids (Gibco-BRL). Flasks were incubated overnight at 22^{o}C to allow explant attachment before exposure to either 0 Gy, 0.1 Gy, 0.5 Gy, 1.0 Gy, 5 Gy and 10 Gy radiation from a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin 6, Ireland) with a dose rate of 1.8 Gy/min at a source-to-flask distance of 80 cm. Explants were removed from the flasks 2 h after irradiation, cut into small fragments (ca. 1 - 2 mm^2) and stored in RNAlater solution (Sigma-Aldrich) at -20 ^{o}C until required (26, 27).

Isolation of Ribonucleic Acid (RNA) and Reverse Transcription

Explants were homogenized and RNA extracted using the Qiagen RNeasy Micro extraction kit following the suppliers' protocols. Final RNA concentrations were measured spectrophotometrically at 260 nm and 280 nm. Reverse transcription of

RNA employed the Invitrogen Superscript III Reverse Transcriptase kit (Invitrogen Corp.). Briefly, 500 ng of RNA from each tissue sample was incubated at 65 ^{o}C for 15 min with 0.5 µl oligo dT_{20}, 10 mM dNTP followed by ice immersion for 5 min. 4 µl of 5 X 1st Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of SuperRNAse Out and 1µl Superscript III were then added. This was incubated at 50 ^{o}C for 60 min followed by enzyme denaturation at 70 ^{o}C for 15 min.

Gene Expression Analysis

Table 6.1 shows the details of all primers employed. Complementary DNA (cDNA) (25 ng) was used in each 20 µl real-time PCR reaction SYBR Green Master Mix (Qiagen) containing 0.5 µM primers. The efficiency of each primer set (E) was determined by preparing a dilution series of template. Relative expression values were calculated following the mathematical expression [$E^{-\Delta\Delta Ct}$] generally used for real time PCR data and originally proposed by Pfaffl (2001) where Ct = the cycle threshold (the number of PCR cycles required before the fluorescent signal is detected above background levels) (28). Each cDNA sample was measured in triplicate for each primer set.

Gene	Origin	Accession No.	Primer F1	Primer R1
β -actin	Nuclear	AJ438158	gaagatgaaatcgccgcactgg	ctttctggcccatcccaacca
Cytochrome c subunit Vb	Nuclear	AF255351	tccgtacctgtacaaccttgcaattg	ttcctttcttcagtgcctgcaagg
Cytochrome c Oxidase 1	mtDNA	NP_008292	ctcaaccaaccacaaagacattggc	tcacgttatagatttggtcatccccc
ATPase subunit 6	mtDNA	NP_008295	cttcttcgaccaatttatgagcccc	tcggttgatgaaccacccttgc
NADH dehydrogenase subunit 1	mtDNA	NP_008290	tagcatacattgtacccgttctgttagcag	aatagttttaggccgtctgcgatgg

Table 6.1 Details of target genes and primer sequences for the gene expression study.

Extraction of Mitochondria

Approximately 1 g of tissue explant was homogenised in 2 ml of ice cold mitochondrial extraction buffer (2 mM EGTA, 20 mM Tris, 0.25 mM Sucrose, 40 mM Potassium chloride (KCl)). The homogenate was centrifuged at 2000 g for 10 min at 6 ^{o}C, the supernatant removed and centrifuged at 10,000 g for 10 min at 6 ^{o}C. The resulting mitochondrial pellet was re-suspended in 200 µl of a 10 % glycerol PBS solution. Earlier work by these authors identified a reduction of enzyme activity following freeze-thaw cycles, particularly in Complex II/III. The inclusion of 10% glycerol negated this effect. Samples were frozen at -80^{o}C until needed for enzyme kinetic analyses. Negligible losses of activity were identified in samples stored at -80^{o}C over the timeframe of this study. Protein measurements of all samples were performed in triplicate using the Bradford assay following enzyme analysis (29).

Enzyme Analyses

The activity of OXPHOS enzyme complexes I, II-III, IV, V were measured following techniques described by James *et al.* (1996) with some minor modifications (30). The assay for citrate synthase (CS) activity, a marker enzyme used to identify intact mitochondrial mass, is based on the original assay performed by Shepherd and Garland (1969) (31).

The measurement of complex I activity is based on the reaction;

$NADH + H^{+} + UQ1 = (I) \Rightarrow NAD + UQ1H_2$

It was measured by preparing 10 mM Tris, 50 mM KCl, 1 mM EDTA, pH 7.4, 2.5 mM NADH, 40 mM KCN, 12 µM antimycin A_1 and 1 mg/ml phosphatidyl choline) and 10 µl of sample and equilibrating to 30 ^{o}C prior to the addition of 10 µl of 0.8 mM coenzyme Q10. The rate of disappearance of NADH was measured at 340 nm ($\varepsilon_{340} = 6.811\ nM^{-1}\ cm^{-1}$)

The measurement of complex II-III activity is based on the reaction;

Succinate + Oxidised cytochrome C = (II-III) $\Rightarrow$ Malate + Reduced cytochrome C.

This reaction was measured by preparing 50 mM KH_2PO_4, 0.1 mM EDTA, pH 7.4, 12 µM antimycin $A_{1,}$ 0.8 mM rotenone, 2 mM reduced cytochrome c in SDW and equilibrating to 30 ^{o}C prior to the addition of 10 µl sample. The rate of reduction of cytochrome *c* was measured at 550 nm ($\varepsilon 550 = 21.1 nM^{-1}\ cm^{-1}$)

The measurement of complex IV activity is based on the reaction;

Reduced Cytochrome C + ½02 + 2H+ + 2e- = (IV) $\Rightarrow$ Oxidised Cytochrome C + H_2O.

The decrease in absorption of cytochrome c was measured at 520 nm ($\varepsilon_{520} = 27.7\ nM^{-1}\ cm^{-1}$) in the reaction containing 200 mM Tris, 10 µM EDTA, pH 7.5, 12 µM Antimycin $A_{1,}$ 0.8 mM Rotenone, 2 mM reduced Cytochrome C and equilibrating to 30 ^{o}C prior to the addition of 10 µl sample.

The measurement of complex V activity is based on two reactions; firstly ATP is hydrolysed to ADP in the presence of Complex V and this ADP is reformed by a reaction with phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (PK) to form ATP and pyruvate. In the second step, pyruvate is converted to lactate by lactate dehydrogenase (LDH) and the oxidation of NADH to NAD is measured at 340 nm ($\varepsilon_{340} = 6.811\ nM^{-1}\ cm^{-1}$).

The reactions are;

ATP $=^{V}\Rightarrow$ ADP + Pi

ADP + PEP $=^{PK}\Rightarrow$ ATP + Pyruvate

Pyruvate + NADH $=^{LDH}\Rightarrow$ Lactate + NAD

This reaction was measured by preparing 2 mM $MgCl_{2,}$ 0.2 mM EDTA, pH 8.0, 2.5 mM NADH, 50 mM MgATP, 40 mM KCN, 0.8 mM rotenone, 100 µM antimycin $A_{1,}$

20 mM phosphoenol pyruvate, 60 units pyruvate kinase, 150 units lactate dehydrogenase and equilibrating to 30 ^{o}C prior to the addition of 10 µl sample and measuring absorbance.

Citrate Synthase

Citrate synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle. The enzyme catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid. This enzyme is an exclusive marker of the mitochondrial matrix. To measure citrate synthase prepare 170 µl of 10 mM Tris Buffer, 2 µl acetyl CoA (15 mg/ml), 20 µl DTNB (5'5'-dithio-bis-(2-nitrobenzoic) acid (2 mg/ml)) and 5 µl sample tissue homogenate was equilibrated to 30 ^{o}C. 5 µl of oxaloacetic acid was added and the rate of absorbance change recorded at 412 nm for 5 min. Protein content of the sample was quantified using the Bradford Assay. Citrate synthase (CS) activity was calculated as nmoles/min/mg protein using the equation A = ecl (ε_{412} = 13.6 nM^{-1} cm^{-1}) to convert dA/min to nmoles per min.

Statistical Analyses

The data presented are mean values ± standard errors for three to five independent experiments incorporating at least three replicates per experiment. All statistical analyses were carried out using the Prism software package (Graphpad Software Inc., CA, USA.). Significance was determined using Anova followed by Dunnett's Multiple Comparison Test comparing treated samples to control. A post analysis test for linear trend, which calculates linear regression on group means versus dose, was carried to determine if dose responses were significant. Differences were considered significant if $P \leq 0.05$.

6.4 Results.

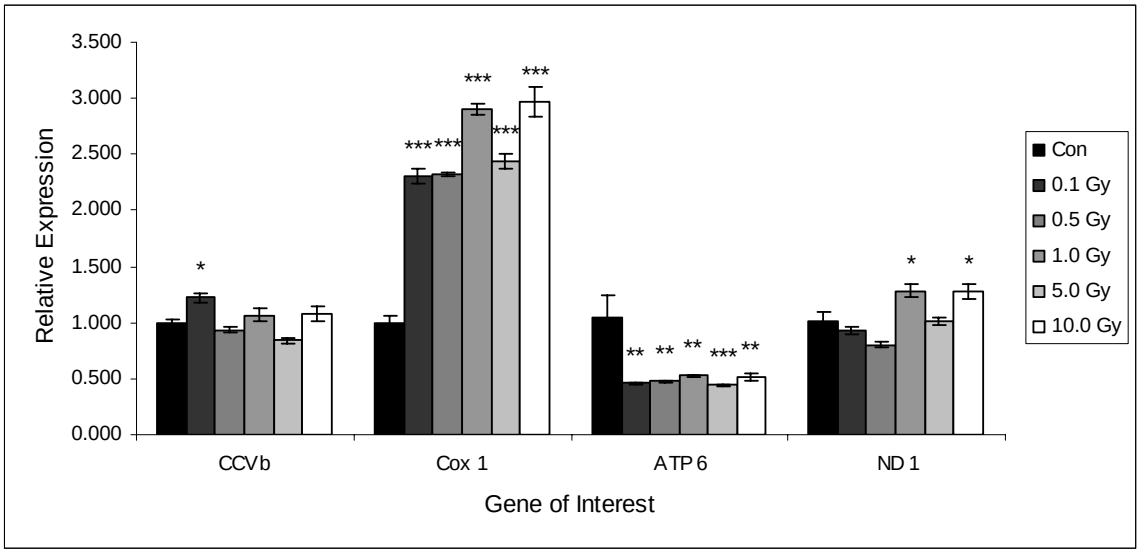
Gene Expression

The effect of irradiation dose on gill tissue expression of the four selected genes is illustrated in Figure 6.1 (a). There is no definitive relationship between cytochrome C subunit Vb (CCVb) gene expression in gill with exposure dose. However, the 0.1 Gy dose did elicit a significant up-regulation in this gene. Conversely, the cytochrome oxidase subunit 1 gene (Cox 1) is significantly up-regulated in all radiation doses. Furthermore, there is an extremely significant linear correlation ($P < 0.0001$) associated with Cox 1 gene expression and increased radiation dose. In contrast, there is a highly significant negative correlation ($P < 0.0022$) with ATPase subunit 6 (ATP 6) gene expression and dose. There was significant up-regulation in NADH dehydrogenase subunit 1 (ND 1) gene expression in the gill tissues at 1 Gy and 10 Gy though not 5 Gy.

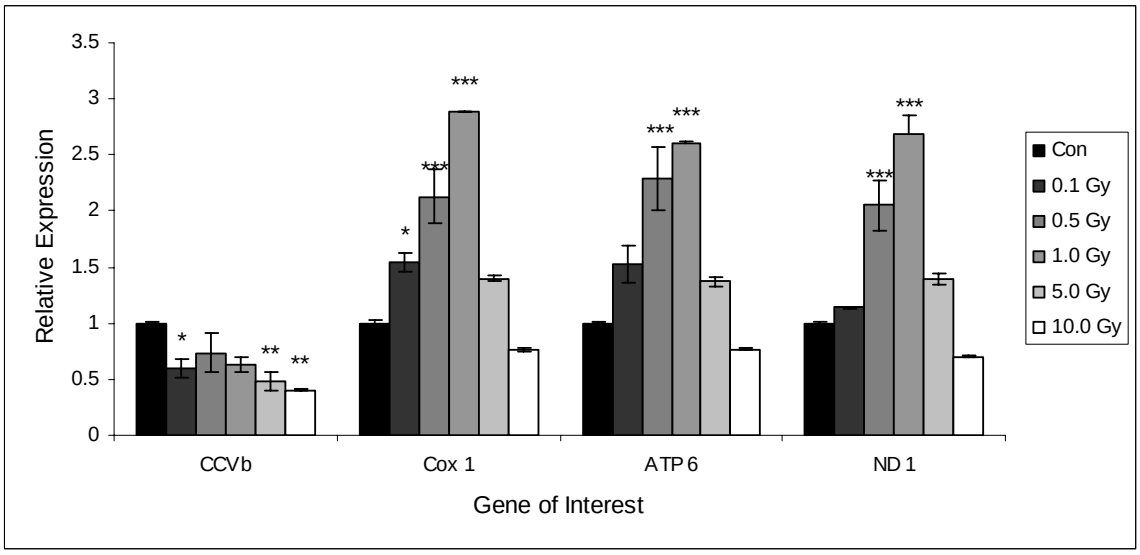
In rainbow trout skin tissues exposed to irradiation *in vitro* (Figure 6.1 (b), the nuclear encoded mitochondrial based CCVb gene shows an extremely significant negative correlation between expression and increased dose exposure. Expression of all the mitochondrially-encoded genes in this study (Cox 1, ATP 6 and ND1) were significantly up-regulated ($P < 0.0001$) following exposures up to and including 1 Gy. No significant change was identified in expression levels in the 5 Gy and 10 Gy doses from control.

Gene expression in spleen tissues was remarkable both for the very large activation in expression of all genes exposed to 0.1 Gy (4 – 14 fold) and for the narrow window of dose that effect this (Figure 6.1 (c). There are no significant differences observed in samples exposed to radiation greater than 0.1 Gy.

(A)


3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
Relative Expression
CCVb
Cox 1
ATP 6
ND 1
Gene of Interest
Con
0.1 Gy
0.5 Gy
1.0 Gy
5.0 Gy
10.0 Gy

(B)


3.5
3
2.5
2
1.5
1
0.5
0
Relative Expression
CCVb
Cox 1
ATP 6
ND 1
Gene of Interest
Con
0.1 Gy
0.5 Gy
1.0 Gy
5.0 Gy
10.0 Gy

(C)

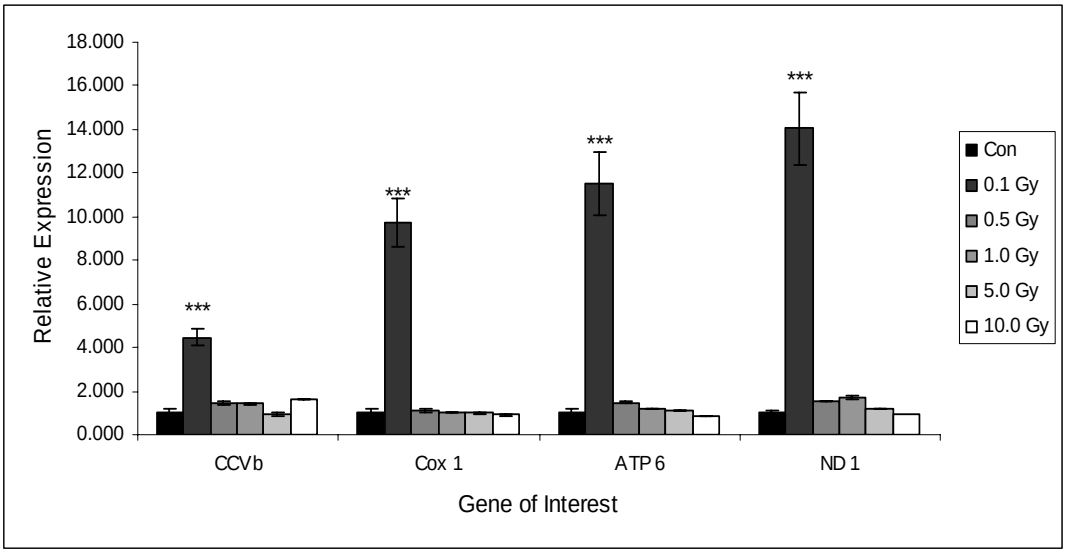


Figure 6.1 Expression levels of mitochondrial related genes, CCVb, Cox 1, ATP 6 and ND1, in gill tissues (A), in skin tissues (B) and in spleen tissues (C). Cycle Threshold (Ct) values are normalized to the mean activity of the housekeeping gene β-actin and expressed relative to the non-irradiated control. Values are expressed as means of 3 independent experiments ± S.E. * denotes *P ≤ 0.05*, ** denotes *P ≤ 0.01*, * denotes *P ≤ 0.001*.**

Enzyme Kinetics

The activity of complex I in gill, skin and spleen is shown in Figure 6.2. There were considerable differences in the basal activity between spleen and the other tissue types. However there are no significant changes in the level of this complex with dose.

Complex II/III (Figure 6.3) showed the highest basal level of activity in spleen tissues, with lesser activity in skin tissue and gill tissue. There was a significant increase ($P = 0.034$) in complex activity in only those gill tissues exposed to 0.1, with activity observed as that of control post higher exposures. Neither skin nor spleen tissues exhibited any marked changes in complex II/III levels following treatment.

Basal activity of Complex IV in spleen was considerably lower ($\geq$ 5 fold) than in either gill or skin tissues (Figure 6.4). However, no significant changes in Complex IV enzyme activity were observed for any of the tissues compared to their respective controls.

Complex V results showed a similar pattern to complex IV in that basal activity in the spleen was much less than either gill or skin (Figure 6.5). Similarly, no marked trend of response to exposure dose was observable in any of the tissues analysed with respect to their controls.

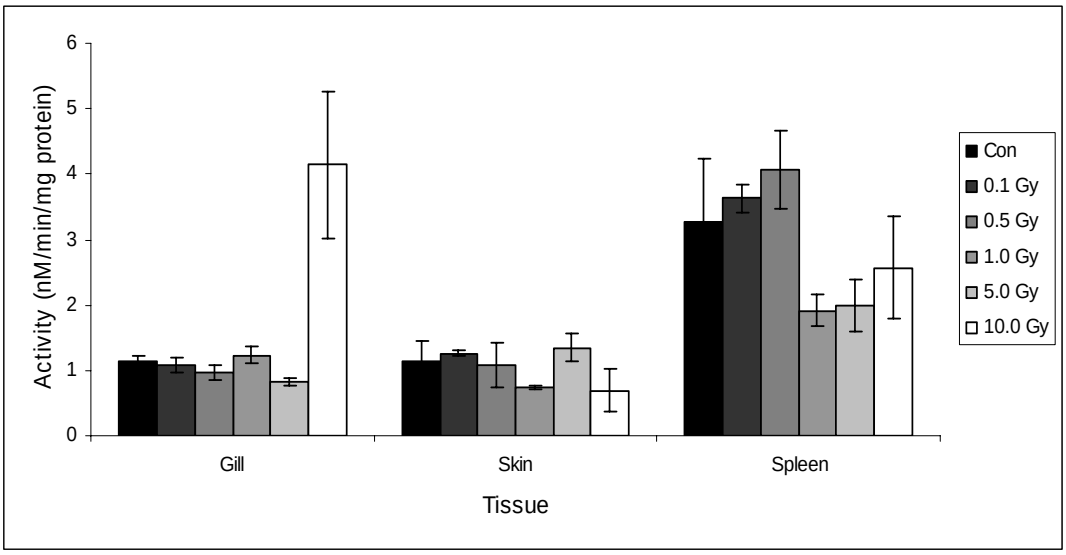


Figure 6.2 Complex I activity in mitochondria fractions of gill, skin and spleen tissue explants following exposure to 60Co ionizing radiation. Values are expressed as a mean of 3 independent experiments ± S.E. * denotes P ≤ 0.05, ** denotes P ≤ 0.005.

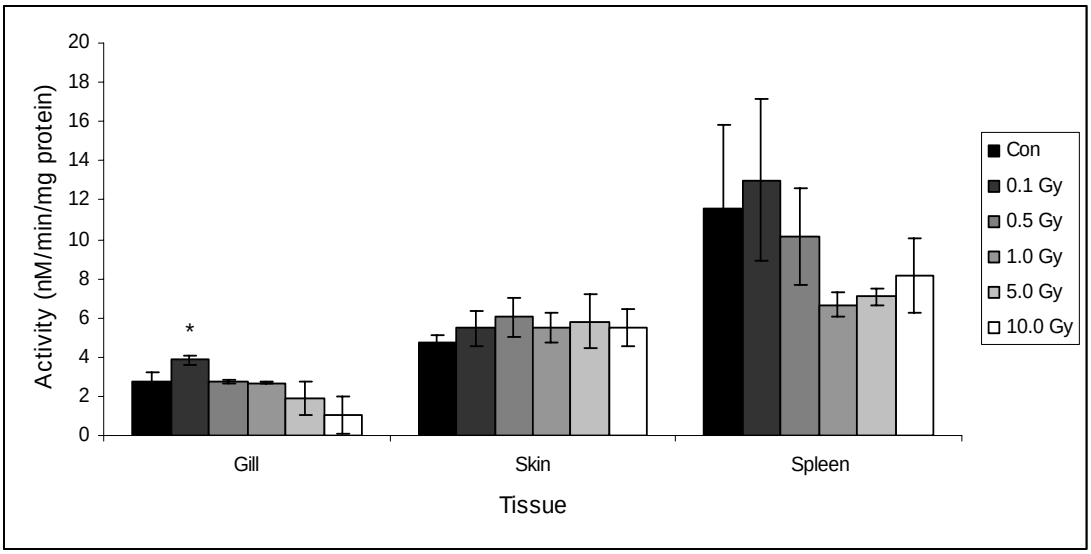


Figure 6.3 Complex II/III activity in mitochondria fractions of gill, skin and spleen tissue explants following exposure to 60Co ionizing radiation. Values are expressed as means of 3 independent experiments ± S.E. * denotes P ≤ 0.05, ** denotes P ≤ 0.01

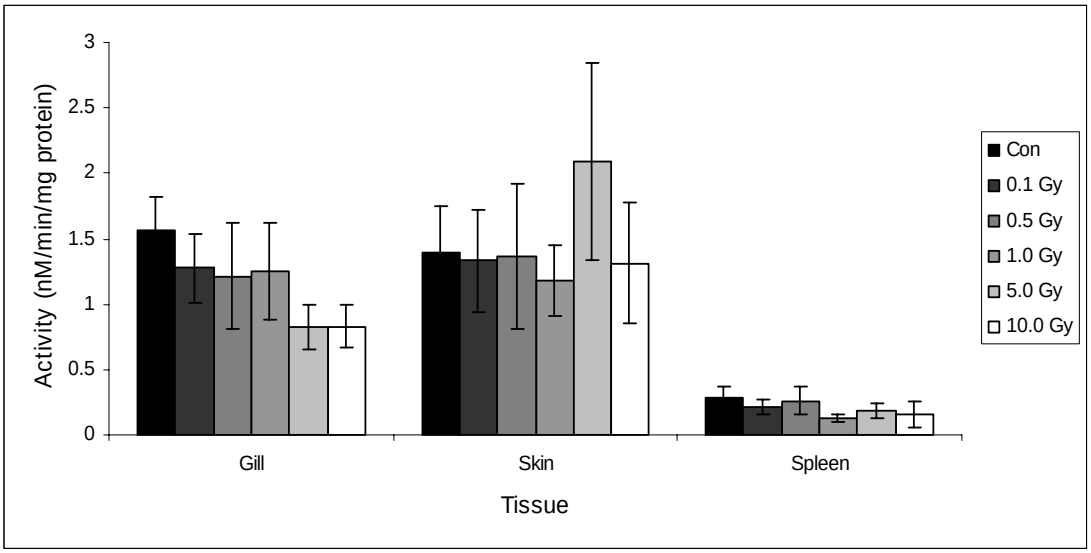


Figure 6.4 Complex IV activity in mitochondrial fractions of gill, skin and spleen tissue explants following exposure to 60Co ionizing radiation. Values are expressed as means of 3 independent experiments ± S.E. * denotes P ≤ 0.05, ** denotes P ≤ 0.01,

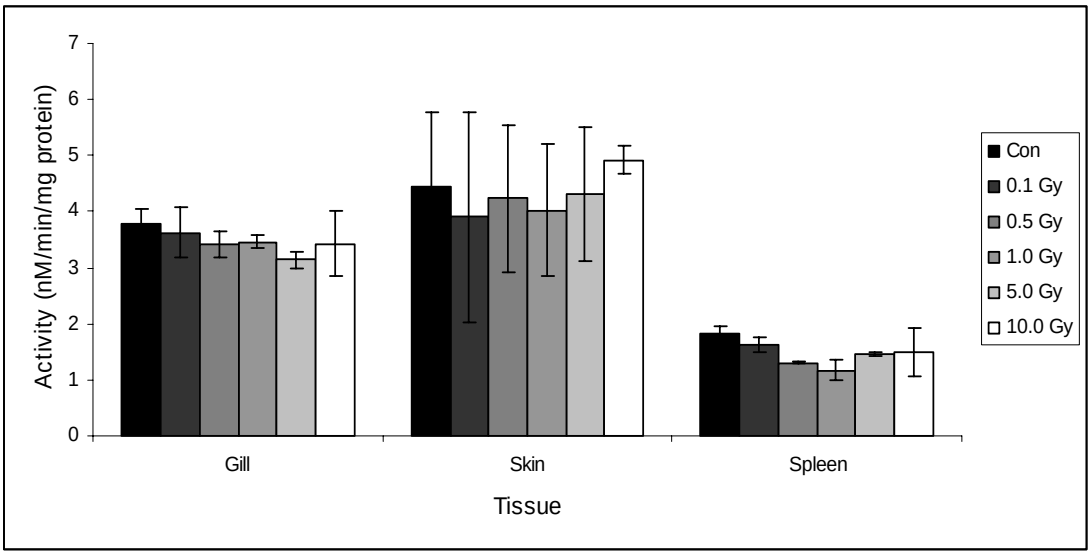


Figure 6.5 Complex V activity in mitochondrial fractions of gill, skin and spleen tissue explants following exposure to 60Co ionizing radiation. Values are expressed as a mean of 3 independent experiments ± S.E. * denotes P ≤ 0.05, ** denotes P ≤ 0.005.

Basal citrate synthase (CS) activity across tissue types showed higher levels of activity in gill and skin tissues relative to spleen (approximately 2.5 times). However, the dose responses were all quite different (Figure 6.6). The CS activity in exposed gill tissues also showed a highly significant and marked loss of activity following 0.1 - 0.5 Gy exposure, however activity was not affected by 1 – 5 Gy doses. Indeed a significant increase was observed post 10 Gy with respect to control. All exposed skin tissues showed a highly significant loss of CS activity compared to the control. Linear trend analysis identified a highly significant ($P < 0.0001$) negative correlation with dose in skin tissues. Interestingly, this analysis also identified the same negative dose effect in exposed spleen tissues albeit exhibiting a lower level of activity in the control than either 0.1 Gy or 1.0 Gy samples. At both 5 Gy and 10 Gy exposures, an extremely significant inhibition in CS activity was observed.

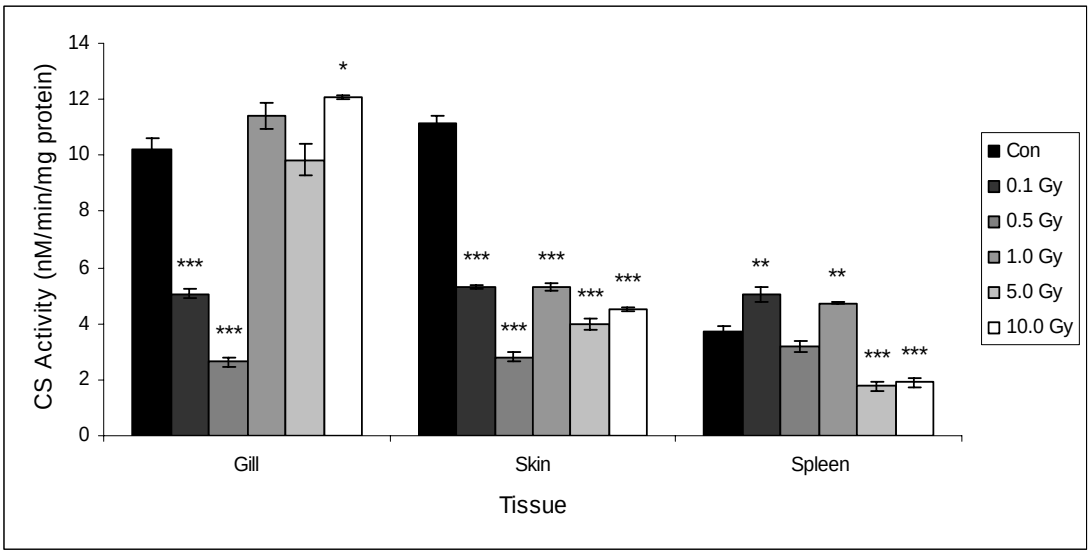


Figure 6.6 Citrate synthase activity in gill, skin and spleen tissue explants following exposure to 60Co ionizing radiation. Values are expressed as means of 3 independent experiments ± S.E. * denotes P ≤ 0.05, ** denotes P ≤ 0.01, * denotes P ≤ 0.001**

6.5 Discussion.

This study focused on the regulation of several mitochondrial encoded genes associated with OXPHOS in *in vitro* cultures of gill, skin and spleen tissues of rainbow trout exposed to γ radiation (Figure 6.7). The CCVb (nuclear encoded) and Cox I (mitochondrially encoded) genes used in this study code for components of the Complex IV enzyme while ATP 6 and ND I genes code for components of complexes V and I respectively (Table 6.1). The expression of these genes in gill, skin and spleen tissue cultures following exposure to γ radiation, were measured relative to ß-actin.

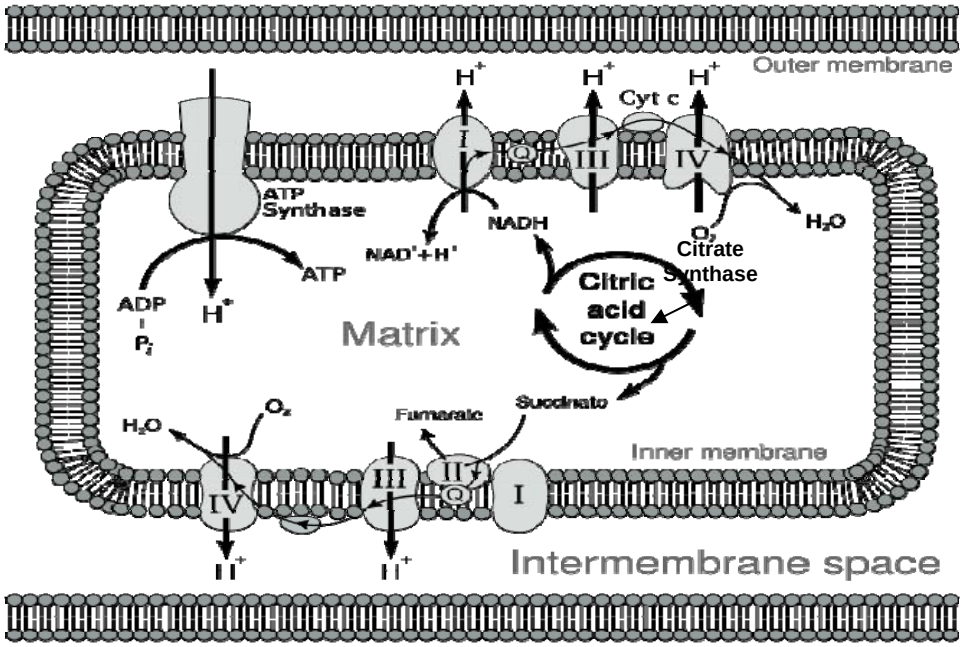


Figure 6.7 Diagram showing the components and reactions of the electron transport chain (ETC). Genes used in this study code for some of the components associated with this system. CCVb is nuclear encoded and codes for Complex IV, Cox I is mitochondrial-encoded and codes for Complex I. The nuclear encoded marker enzyme for intact mitochondria, citrate synthase, is also shown as part of the citric acid cycle which subsequently generates 3 NADH and $FADH_2$ to ferry electrons to the electron transport chain.

Results from gill and skin tissues showed some similarities. The nuclear encoded CCVb gene is generally down regulated in both gill and skin while the mitochondrially encoded Cox 1 gene is up-regulated in both. This is not the case in the spleen tissues where there is a general, though not universal, up-regulation of the CCVb gene and no change in the Cox 1 gene, with the exception of the 0.1 Gy treatments. This is interesting in that both of these genes code for the Complex IV enzyme of the Electron Transport Chain (ETC). One would expect that if one gene is regulated to code for a particular protein complex, then another gene associated with that complex is regulated in the same way.

The Cox 1 gene also behaves differently at higher doses in both gill and skin tissues. While the Cox I gene continues to be up-regulated in a dose dependent manner up to 10 Gy in the gill tissue, skin tissues display a threshold limit at 1 Gy and beyond where Cox I gene activity returns to control levels.

This significant trend is also identified in the ATP 6 and ND 1 genes in skin tissues. This suggests that skin tissues in rainbow trout have a radiation threshold limit which alters the regulation of mitochondrial genes coding for key enzymes associated with the ETC. This is not clear in the case of gill. The ATP 6 gene results show no changes in regulation following treatment while the ND 1 gene shows some up regulation but only at the higher doses.

The relative expression of genes in spleen tissues deviates considerably from both gill and skin above. The significant up-regulation of all the genes in spleen tissues at the 0.1 Gy treatments suggests that within this tissue, a similar threshold exists as in the skin tissue. However it is activated at a dose one order of magnitude less than skin and is considerably more radiosensitive evidenced by the narrow window within which this up-regulation occurs. In addition, the up-regulation of the CCVb gene in

spleen tissues, which is nuclear encoded but mitochondrial associated indicates that the threshold effect deviates somewhat in how it is activated in both tissues types.

Generally, there appears to be little correlation between gene expression results and the activity of complexes in this study. There may be several reasons for this. The 2h interval post-treatment may have affected this. While Lyng *et al*. (2001) found calcium fluxes in mitochondria just 30 seconds following exposure to irradiated cell conditioned media in human keratinocytes, a considerable 'time-delay' may exist between the assembly of the genetic machinery and the generation of enzyme complexes for utilisation within the mitochondria (32).

Within many of the enzyme complex results, rather large errors exist which prevent statistically significant differences being achieved. This appears to be an issue throughout studies relating to fish. The incorporation of at least 5 replicates would certainly be recommended in this case.

The enzyme kinetic results do identify an interesting pattern where gill and skin tissues are comparable in their general levels of activity relative to spleen. In some ways this is similar to the pattern found in the gene expression study. Specifically, this manifests as having low relative activity in complex I and II/III and high relative activity in complexes IV and V. Indeed it is clear that in mammalian systems at least, the ratios of complexes are not equal. A commonly used ratio in mammalian systems proposed by Hatefi (1985) for complexes I:II:III:IV:V is 1:2:3:7:4 (33). More recently Schagger and Pfeiffer (2001) suggested a ratio of 1:1:3:7:4 in the bovine heart (34). This ties in well with the levels of activity found in this study for gill and skin. However spleen results would contradict these.

On the basis that there are no significant changes in any of the enzyme complexes in skin tissues exposed to a wide range of radiation doses, one could conclude that the

tissue is quite radio-resistant. However, as Figure 6.1(b) shows, considerable changes are occurring at a genetic level in both nuclear and mitochondrial encoded genes. This may suggest that the measurement of enzyme activity in fish skin tissues may not be the most sensitive tool to identify toxic stress in fish. This however may be limited to radiation exposure. Many suggest that high levels of melanin, which rainbow trout skin tissues contain, confer significant resistance to the effects of radiation (35, 36).

CS is commonly used as a quantitative marker for mitochondrial mass (37). CS is localised to the mitochondrion and is encoded exclusively in the nuclear genome. This enzyme assay was included to identify alterations in levels of mitochondria relative to the nuclear component in each sample. The results confirm the generally accepted premise that tissues such as gill and skin, which have a functional role in oxygen supply, have higher levels of mitochondria as measured by CS activity than tissues such as spleen in this case. One can see from the results here that considerable variations exist not only in treated samples but also the control tissues. Moreover, CS assay represents quite a sensitive biomarker of low-level toxic stress in all tissue types with significant differences from control following the lowest exposure doses used.

The level of CS activity in the treated spleen samples appears unpredictable from dose to dose. While some inconsistency exists in the treated gill samples it represents more of a dose threshold type effect than volatility. Conversely, in terms of relating dose to compromised mitochondria, skin tissues show that a significant negative dose response exist confirming regularity in this tissue type.

To summarize, both skin and spleen tissues appear to identify a threshold level of toxic exposure above which the regulatory activity of mitochondrial associated genes is stimulated. The threshold in spleen is very low at 0.1 Gy while the threshold in the

more robust skin is higher at 1 Gy. Differences in gene regulation may be associated with variances in the levels of complexity and functions of the tissue in question. The regulatory trends of the same genes in gill tissues are less consistent and may relate to the gills' interfacing function with both the environment and circulatory systems.

Whereas the gills are exposed to both internal biochemical and externally to gas exchange differentials, pathogenic organisms and toxic substances including free radicals, the skin plays an important role in gas exchange and its prime function is as a physical barrier. While substances such as air pass through it, the skin itself may not be exposed to the perilous biochemical and environmental factors associated with the gill. Moreover, the skin of rainbow trout contains high levels of melanin which confer a certain level of resistance to radiation exposure. The spleen, on the other hand, does not interface with the environment directly and therefore may be more sensitive to 'extra-ordinary' exposure or interference. Whatever the reasons, it is clear that radiation exposure not only stimulates altered responses at different doses but also in different tissue types.

Little correlation could be found between gene regulation and enzyme activity at 2 h post radiation: possibly such correlation could be achieved by sampling at a later time point. A time-series of sampling post treatment over a 24 h period is recommended to identify a more relevant sampling point. Furthermore, an increase in the number of replicates is also recommended given that fish often show quite diverse responses. While CS activity is necessary for the standardisation of mitochondrial mass in tissue samples, results in this study suggest that it may indeed be a sensitive biomarker of low dose radiation in its own right.

Nonetheless, significant alterations in levels of expression in *ex-vivo* fish tissues at low doses of radiation suggest that this is a promising approach for use as a

biomarker in toxicity studies. Moreover, the technique has the potential to significantly reduce the number of live animal experiments with a consequent alleviation of the ethical, moral and economic pressures which currently exist in the area of environmental toxicology.

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CHAPTER 7. GENERAL DISCUSSION.

There is a growing need for *in vitro* technologies for use in the assessment and monitoring of the aquatic environment, and to reduce the current dependence on the use of whole organisms. An increase in the pressure on the aquatic resource in terms of human requirements, commercial exploitation and increased recreational use means that the resource is susceptible to increased levels of pollution and damage.

The aquatic environment is a complex convergence of biological, chemical and physical processes. The ability of an aquatic resource to withstand natural and anthropogenic pressures is dependent on its size, location and stage of development. Therefore few aquatic environments can be treated with a standardised approach. Monitoring and assessment of these resources are therefore very difficult to conduct.

Existing and conventional approaches to monitoring/assessing the aquatic environment typically involve exposing a battery of test organisms to samples from the aquatic environment and / or chemicals which are thought to be linked to the resource and subsequently measuring a 50% lethal concentration or dose level (LC_{50} or LD_{50}).

This approach has served well to date and has been useful in establishing toxic levels in particular environments. However, there are shortcomings and one in particular is that countless organisms, which we are aiming to protect, are being sacrificed annually using these tests. In the past number of years there is increasing interest in the use of *in vitro* based model systems which can identify toxicity in particular samples but do not rely on live organisms to provide lethal dose results.

While this is a worthy cause there are concerns that these model systems do not truly represent the organisms which they are replacing and by continuing to culture these tissues *in vitro*, some of the traits and sensitivities are being lost.

In an effort to address these disparate problems, and achieve a middle ground, this thesis examined the development of toxicity tests based on *in vitro* tissue culture platforms using aquatic organisms. The rationale behind this approach is to significantly reduce the numbers of live animals used in toxicity tests but maintain the direct relationship with the environment being tested. Furthermore, as is shown in Chapter 2, there are inherent and systemic problems in establishing consistent cultures that are adequately robust to provide a repetitive test platform necessary for toxicity monitoring. This is particularly true for invertebrate cultures and especially marine invertebrate cultures, which are unreliable. In addition, no established cell line has been developed from a marine invertebrate organism. In chapter 2, these difficulties and time constraints re-focused the author's direction on establishing a tissue culture as a 'holding and exposure' approach rather than a tissue culture approach followed by an exposure. This facilitates the possibility for the development of an expeditious test system. By focusing on the mitochondrion, which is of prime importance in the cell, and monitoring its function and the mtDNA genome, a practical approach to address the tissue culturing of aquatic organisms and assessment of toxicity was possible. The 'holding and exposure' approach facilities significant reductions in the numbers of fish required for environmental exposure studies. For example, a typical statistically relevant test with a control and 3 exposures (0.5 Gy, 5.0 Gy and 10 Gy) would require a 36 fish while the application of an *in vitro* test using the holding and exposure test requires tissues from 12 fish. This significantly

reduces the numbers of fish required in experimental tests and supportive on both moral and economic grounds.

While ethical concerns are not completely alleviated the technique is a significant move in the right direction in terms of reducing live animal trials and may form a basis for further significant reductions. For example, the culture and exposure of tissues which does not require the sacrifice of animals could bring about further significant improvements in current live animal testing practices. In vitro culture of fin clippings may be a good starting point in this approach.

While the ethical concerns are of foremost concern for many, the economics of eliminating the use of live animals for environmental testing and monitoring will undoubtedly be the basis for bringing an end to these practices. If we consider the costs associated with live animal testing, it is clear that significant cost reductions can be achieved using a suitable in vitro model system. Not only are there significant cost savings due to the reduced number of animals used but also the costly holding and maintenance of animals can be significantly reduced.

To examine this approach and its benefits/malefits further, a stress model was required. A model toxicant system, which was well defined, that caused DNA damage, was easily quantifiable, easy to access and safe to use were identified as the ideal qualities. The application of Co^{60} γ radiation as the stress model for toxic exposure was used, as it fulfilled these requirements. In initial studies, work focused on the investigating the application of a platform, based on fish tissue cultures, which could be used to identify the effects of radiation exposure on these cultures. The initial studies initially focused on molecular aspects associated with the assay system especially the quantity and quality of mtDNA which could be recovered from test samples following exposures. Results, highlighted in Chapter 2 show that good

quality DNA can be recovered from samples held and exposed in the test platform, which facilitates an additional avenue of analysis to be conducted based on PCR. Preliminary results also identify an alteration in the quantity of mtDNA associated with the tissue samples following exposure and this was dependent on tissue type and radiation dose. The possibility of using, this platform and these features, as marker systems of toxic stress were investigated in subsequent chapters.

Chapter 3 investigates standard PCR and molecular techniques to assess the quantity and quality of DNA extracted from fish tissue samples exposed to radiation. The hypothesis being tested is that alterations in quantity and quality of mtDNA could be used as a putative biomarker of toxic stress. In these studies, citrate synthase is used as a marker of mitochondrial mass. Increases in the level of mt genomes did not correlate directly with increases in radiation dose however, some anomalies were highlighted which could be incorporated into a useful model system.

In order to develop the application of radiation as a test stressor and the *in vitro* tissue culture system, a section of the study focused on the bystander effect brought on by exposure to growth media of irradiated tissues. This study was conducted by exposing EPC fish cell cultures to irradiated tissue conditioned media (ITCM) in the clonogenic assay platform. Results from this study are shown in Chapter 4 and published in Radiation Research (1). This study identified that there is a bystander effect in EPC cells exposed to media from radiation exposed rainbow trout tissues. However, a clear difference in response is evident depending on the tissue type. The results show that there is little response in the growth of indicator EPC cells grown in media from irradiated skin tissues, a significant reduction in the growth of cells exposed to ITCM from irradiated gill tissues and an increase in the growth of indicator cells exposed to media from irradiated spleen tissue. The conclusions drawn

from this study identify possible links between attachment and effect, which may compromise the basis of the clonogenic assay in this case. The work suggests that rather than a deleterious effect on the cells directly, the bystander effect displayed is a result of adhesion factors such as ICAM, VCAM and E-selectin into the growth media from the irradiated tissues. These adhesion factors modulate the growth effects of the indicator cells in the subsequent exposures.

In an effort to investigate the cell attachment in clonogenic assays further and to investigate other inconsistencies relating to changes in colony size in irradiated cultures, image analysis was introduced into the work programme. The results of this study are shown in Chapter 5. The aim of the study was to identify any changes in the size of the emerging colonies or indeed the size of the cells in these colonies following exposure to direct and indirect radiation. A wider dose exposure regime was used in these studies to capture the effects of radiation dose rather than effects evident over time following exposure. The conclusions identified here suggest that image analysis in tandem with the clonogenic assay can add value to that assay, not only by establishing the fifty cell colony limit but also identifying changes in the morphology and size of cells and colonies following toxic exposures. A PCR step was also introduced to confirm that the alterations in colony size were not associated with contaminated or co-cultured organisms.

The work shown here in Chapter 6 takes features of previous chapters and brings them a step further to develop an *in vitro* based system suitable for use in aquatic tissue culture systems. Following the confirmation that good quality RNA was extractable from tissues held in culture flasks overnight, irradiated and frozen as shown in Chapter 2 and that the mitochondrial genome is affected by irradiation in fish tissues in Chapter 3 the application of gene expression in irradiated fish

mitochondria offered a very interesting biomarker platform possibility for use in *in vitro* toxicity testing. It also offered the possibility to investigate the effects of multigenic transcription, which is associated with the mitochondrial genome, and how the mitochondrial proteins can be modified independently of each other. Devin *et al.* (2004) suggests that mitochondrial transcription is more dynamic in nature (2). The results shown in Chapter 6 certainly support this as alterations in the mitochondrial genes tested varied depending on tissue types and the dose exposure regimes.

To conclude, there is an unmet need for an *in vitro* based assay system which deviates from the traditional approach of calculating the LD50/LC50 based batteries of live animal testing from representative trophic levels in nature. Many authors have recognised the benefits of using *in vitro* cell culture-based test systems which would avoid the use of live animals. However, problems exist with this approach especially the relationship with the actual organism, the environmental relevance of cell lines and drift in the cell line being cultured over several generations in plastic flasks. As an alternative to cell cultures, others have proposed the use of tissue culture (primary or secondary) as a solution to this problem. While the number of live animals is significantly reduced using this approach, establishing and maintaining tissue cultures can be time consuming, costly and often gives variable results.

The main conclusions of this thesis are as follows:

- The development of a 'holding and treatment' tissue culture platform for use in *in vitro* assays significantly reduces the use of live animals in environmental toxicology testing but maintains the relevancy of the assay due to the primary nature of the cells comprising the explant (Chapter 2).

- The mitochondrion is an ideal marker of toxic stress which can be used *in vitro*. The organelle offers several endpoints including functional activity, mtDNA integrity, mtDNA quantity and several enzyme markers including citrate synthase (Chapter 3).
- Tissue cultures of rainbow trout, which have been irradiated, induce a bystander effect in EPC cells *in vitro*. This effect is dependent on tissue type (Chapter 4).
- The effectiveness of the clonogenic assay may be offset by adhesion factors released from cells following exposure to radiation. Added value can be achieved in the clonogenic assay if used in conjunction with image analysis (Chapter 5).
- Gene expression analysis of tissue cultures using the 'holding and treatment' system, outlined above, is a viable alternative to live animal testing (Chapter 6).

There are numerous possibilities for the development of suitable *in vitro* based technologies which could form the basis for the development of effective *in vitro* based assay platforms. This thesis highlights aspects of the biological systems such as the mitochondrion, DNA, RNA and enzyme kinetics to identify the effects of toxic stress on biological systems. However, time restrictions and financial constraints limited the further development of possibilities identified here. This is especially true for the image analysis section which could have benefited from a more extensive study. Ideally, a study of the adhesion factors released into the ITCM could have shed light on several aspects of this study. Furthermore, the application of gene expression analysis offers significant opportunity to develop additional low cost, accurate and expeditious assays. Gene expression is likely to play an increasing role in environmental toxicology in future development in this area of research as it offers improvements in some, if not all, features of a well-defined biomarker test system

outlined above. The development of suitable microarray chips associated with organisms from the aquatic environment would facilitate major advances in this area. The mitochondrion shows major potential in the area of toxicology and should be pursued. It is felt that this organelle offers many opportunities for the development of *in vitro* based applications in environmental toxicology. Mitochondria have been overlooked for too long in this respect. Indeed this work would also have benefited from the development of a mitochondrial gene array which could be applied in the same way as microarrays are currently being used. However, with only 13 genes, the 'mitoarray' would be a more elegant system if proven successful.

Notwithstanding these issues, the work presented here outlines practical aspects and alternatives which should be considered in the future development of environmental toxicology. It offers the potential for significant reductions in live animal testing while maintaining relevancy to the specific environment being tested and highlights issues relating to radiation biology and the bystander effect which have received little attention to date in the aquatic environment.

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