



Society for Experimental Biology Annual Main Meeting
28th June – 1st July 2009, Glasgow, UK

A4 – REPLACEMENT OF MAMMALIAN MODELS: THE ROLE OF IN VITRO TECHNIQUES WITH FISH AND INVERTEBRATES

A4.1

09:00 Sunday 28th June 2009

Tom Hutchinson (PML, UK)

To be confirmed

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A4.2

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Vicki Stone (Napier University)

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A4.3

10:10 Sunday 28th June 2009

The zebrafish heart – A suitable model for human cardiology?

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The zebrafish is a tropical teleost fish that has been the focus of an increasing number of developmental studies. Our recent data suggests that the adult zebrafish may be a suitable model for investigating human cardiomyopathies. Using freshly isolated ventricular myocytes from adult zebrafish we measured action potentials, sarcolemmal ion currents and cellular Ca transients. Our aim was to characterize the electrophysiological properties of the adult myocyte and compare it to that of humans. I_{Ca} density was $-9.58 \pm$

1.8 pA/pF at 0 mV and the time to reach 37% of I_{Ca} peak was $29.2 \pm 2.6 \text{ ms}$ ($n=7$) which are similar values to those of mammals. I_{Na} density was $-84 \pm 15 \text{ pA/pF}$ at -40 mV ($n=5$) which is about 4-times less than that of most mammals. At 0.1 Hz, resting membrane potential was $-70.4 \pm 2.8 \text{ mV}$ and APD₂₅, APD₅₀, and APD₉₀ was 48 ± 14 , 112 ± 23 , $151 \pm 30 \text{ ms}$, respectively ($n=14$). The action potential configuration with a prominent plateau phase closely resembles that from large mammalian ventricular myocytes, notably human. Increasing stimulation rate to 1 and 2 Hz significantly decreased APD₅₀, and APD₉₀. This feature is also characteristics of large mammals. Together these results indicate that adult zebrafish cardiomyocytes is ideally suited for investigation of ion channels related mutation screening of cardiac alteration important in human.

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A4.4

11:00 Sunday 28th June 2009

Isolated liver cells and perfused hearts reveal glycerol management strategies in freeze resistant rainbow smelt

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Rainbow smelt (*Osmerus mordax*), a small anadromous teleost, accumulates glycerol in plasma and all other tissues to levels in excess of 300 mM in response to winter temperatures. High glycerol level contributes to the freezing point depression that allows this species to survive at the freeze point of sea water. Enzyme activity profiles, glycogen levels, feeding studies, and injection of fish with isotopes reveal that glycerol is produced in the liver from carbohydrate and amino acids that are either stored or dietary. Glycerol produced in the liver is subsequently delivered to and taken up by other tissues. Isolated liver cells from fish acclimated to

warm temperature (8 °C) and incubated at warm temperature release glucose but incubation at low temperature (0.4 °C) results in the equimolar release of both glucose and glycerol. A decrease in temperature alone is therefore sufficient to trigger the glycerol production mechanism by isolated cells. Adrenergic stimulation does not stimulate glycerol production and glucocorticoid has no effect beyond that resulting from low temperature alone. Isolated liver cells from fish acclimated to low temperature produce only small amounts of glycerol at low incubation temperature. Isolated perfused hearts take up glycerol via simple diffusion up to extracellular levels of 300 mM glycerol. Acclimation to low temperature does not influence the rate of glycerol uptake and aquaglyceroporins do not appear to be involved in uptake. These findings are potentially relevant to human medicine as the management of glycerol is the target of recent treatments for type II diabetes.

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A4.5

11:35 Sunday 28th June 2009

Using rainbow trout cardiomyocytes to identify the diffusion restrictions found specifically in oxidative muscles

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Studies comparing permeabilized fibres and isolated mitochondria have shown that intracellular diffusion of ADP and P_i is restricted in oxidative, but not glycolytic muscles. This is particularly interesting in the case of the heart, because the compromised energetic balance caused by ischemia–reperfusion is associated with diminished diffusion restrictions. Thus, diffusion restrictions seem to be a prerequisite for the tight regulation of energy production by mitochondria to match energy consumption by ATPases. Despite decades of research the factor(s) that cause diffusion restriction is still unknown. Possible candidates are intracellular membrane structures such as t-tubules, sarcoplasmic reticulum and outer mitochondrial membrane. Most experiments have been done on mammalian cardiomyocytes, but skinned fibre experiments suggest that diffusion restrictions also exist in heart of rainbow trout. However, fibres are many times thicker than isolated cardiomyocytes, which may lead to artefacts. Therefore, we compared for the first time permeabilized cardiomyocytes and isolated mitochondria from heart of rainbow trout to show that diffusion restrictions are indeed present in rainbow trout cardiomyocytes. This validates our use of rainbow trout cardiomyocytes to replace rat cardiomyocytes in many of our experiments. We show by deconvolved confocal images how simple the structure of trout cardiomyocytes is compared to rat cardiomyocytes. Beneath the sarcolemma, a single layer of myofilaments surrounds a central core of mitochondria. This makes rainbow trout cardiomyocytes ideal—maybe even better than rat cardiomyocytes—for our further studies to localize the diffusion restrictions by raster image correlation spectroscopy and mathematical models of regulation of mitochondrial respiration.

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A4.6

11:55 Sunday 28th June 2009

Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1

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Pharmaceuticals and personal care products (PPCPs) are an extraordinarily diverse group of chemicals used in veterinary medicine, agricultural practice, human health and cosmetic care. The toxicological implications of the presence of PPCPs in the aquatic environment remain largely unknown. Acute toxicity tests have generally failed to detect the subtle action elicited by those compounds at environmentally relevant concentrations, and they have often overlooked the fact that toxicity can be influenced by additive and synergistic effects. The aim of this study was to further assess the cytotoxicity of pharmaceuticals from different therapeutic classes and synthetic musks, namely nitro- and polycyclic musks, as well as their mixtures on the rainbow trout liver cell line RTL-W1. Two fluorescent dyes were used to monitor cell viability. Among the tested compounds, estimated EC₅₀s (effective concentration causing 50% decline of cell viability) denoted that polycyclic musks (7–25 μM) followed by antidepressives (7–50 μM) showed the highest potential to induce cytotoxicity, whereas lipid regulators (20–380 μM), anti-inflammatory drugs (160–260 μM) and nitromusks (100–240 μM) had the lowest toxicity. Within a given therapeutic class, combined toxicity of mixtures was additive, following in most cases the concentration addition concept. However, the combined toxicity was higher than additive for those mixtures that included one compound from each class (i.e. dissimilar mixtures). Overall, this study shows that in the aquatic environment, toxicity of PPCPs on non target organisms may occur at concentrations lower than expected due to synergistic effects between the different toxicants.

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A4.7

Poster Session - Monday 29th June

Activities of Spanish network for alternatives (REMA) in promoting animal alternatives

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The Spanish Network for the Development of Alternative Methods (REMA) is a platform that coordinates the initiatives of the Industry, the Administration, the Society and Academia towards a reduction and a more rational use of laboratory animals promoting the development of alternative methods.

REMA is organizing courses on different “hot” topics since 1999 (REACH, Genomics, Bottlenecks on 3R's for pharmaceuticals etc) giving expert opinion by requirement of different regulatory agencies (revision of 86/609 etc) and participating EU projects (SSA) to transfer technology and knowledge on alternatives.

In vitro replacement tests has been developed both by academia and industry with the support of the European Commission, and the result is an important number of scientifically sound methods and new

strategies. However, the transfer of these inventions to potential users has been much slower than expected, mainly due to difficulties encountered in the transferability, official approval as well as production of test kits under conditions of regulatory requirements. The present gap between inventions and potential users needs to be bridged. REMA is a partner in the EU funded project “For Invitox” which analyses the applicability of results of EC funded projects and aims to bring inventors (researchers), users (industry) and producers (manufactures, technological companies) together and establish a Forum facilitate to contacts with regulatory agencies continuously and in this way speeding up the process of making *in vitro* methods available for end-users.

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A4.8

13:45 Sunday 28th June 2009

Has the 3R's concept a future in ecotoxicology?

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The 3Rs approach as formulated by Russel and Birch in 1959 is outlining 3 strategies for reducing the number and the suffering of experimental animals used in research and testing through “Replacement, Reduction and Refinement”. We here discuss if a 3R's based approach can be applied in testing of ecotoxicity of chemicals with the ambition to abolish the use of experimental animals in favour of a battery of alternative methods. The current status of alternatives in aquatic ecotoxicology is reviewed and how well they perform in comparison with current *in vivo* methods. We conclude that theoretically can alternative methods and approaches replace animal based testing but the way to reach this goal is long. A development of more sophisticated alternative methods is needed focusing on specific and physiologically/toxicologically representative endpoints. We underline the importance to gain more information on toxic mechanisms of chemicals and here will hopefully the rapid developments in the “omics” area give new possibilities. The issue is how to interpret results from highly refined *in vitro* systems to “ecological relevance”. The leap is probably not as big as it seems in a first glance. We should not be blinded by ecological “fundamentalism”. The ecosystem is built up by individuals. If the survival and fitness of individuals are compromised the whole system will suffer. The current *in vitro* methods and particularly and hopefully the future methods have the full potential to protect individuals, and by proper models and assumptions the ecosystem.

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A4.9

14:20 Sunday 28th June 2009

Potential of mussel gill cell primary cultures for toxicity testing of conventional and emerging environmental pollutants

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Marine invertebrate cells can be used in *in vitro* toxicity testing to assess the potential risk of environmental pollutants on marine and estuarine ecosystems. The aim of this work is to present data on

the use of mussel *Mytilus galloprovincialis* gill cells as a useful tool for toxicity testing of environmental pollutants, complementary to *in vivo* studies. Mussels are filter-feeding bivalve molluscs that greatly accumulate pollutants in their tissues and are used worldwide in monitoring biological effects of pollutants. Gills are in direct contact with pollutants dissolved in water or associated to food particles, thus constituting one of their first targets. Cells comprising the gill epithelium of mussels have recently been characterized *in vivo* and *in vitro*. Gill cell suspensions obtained through dissociation with dispase are heterogeneous but ciliated epithelial cells are predominant. A battery of *in vitro* techniques has been developed for mussel gill cells including general toxicity tests (cell viability), lysosomal enzyme activity, multixenobiotic resistance MXR transport activity and reactive oxygen species (ROS) production. The suitability of the battery was tested in a series of experiments where mussel gill cells were exposed *in vitro* to cadmium, copper, mercury and benzo(a)pyrene. With the aim of determining the *in vitro* effects of metallic nanoparticles, preliminary experiments were carried out comparing metals in bulk form versus nanoparticles. Finally, *in vitro* responses of mussel gill cells are compared with those of other respiratory cell cultures such as mammalian lung cells. Overall, *in vitro* tests with mussel gill cells provide relevant mechanistic data on adverse effects of environmental pollutants and could have potential for future implementation in regulation (e.g., REACH).

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A4.10

14:55 Sunday 28th June 2009

Development of microfluidic-based Lab-on-a-Chip devices with fish cell lines as biosensors for aquatic contaminants

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Many chemicals, natural or manmade, accidentally or purposely discharged, end up in water bodies, causing adverse effects to the aquatic biota and ultimately to humans. Therefore, monitoring of water sources for toxicants or potential toxicants is of high priority. Regulatory testing of industrial effluents often requires fish lethality assays which can be laborious and costly. Thus, alternative evaluation methods that are simpler, faster and cheaper but still sensitive and relevant have been sought for the testing of effluents or of individual chemicals. This is especially relevant when the safety of drinking water sources is becoming compromised. Portable devices that can provide results in real time and with high throughput are being developed but most involve using genetically modified bacteria or invertebrates and might be difficult to extrapolate to vertebrates. Fish cell lines are convenient model vertebrate cells for evaluating aquatic contamination. Unlike mammalian cell cultures, fish cells can be maintained at room temperature without the need of incubators and can withstand wider pH and osmotic fluctuations, thus are useful cell models for developing aquatic biomedical devices. In this study, we report on the development of microfluidic devices that are coupled with fish cells to detect model chemical contaminants. These biochip devices are based on methods developed for larger scale cytotoxicity assays, which are then miniaturized as very small chambers within a flow-through microscale perfusion device contained within a microscope slide-like unit. Portable biochip devices could be

extremely useful for detecting and evaluating toxicants in drinking water.

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A4.11

Poster Session - Monday 29th June

The use of zebrafish (*Danio rerio*) embryos in a high definition transcriptomic expression profiling approach to ecotoxicological investigations

Ashley D. Sawle (University of Liverpool), Andrew R. Cossins (University of Liverpool)

The adoption of alternative models is often hindered by the limited scope and definition of the scientific outputs. The zebrafish embryo offers the convenience and ethical status of an *in vitro* model with the benefits of an integrated understanding of system-wide processes that comes from the assessment of whole organism responses. We have developed a strategy for an intensive assessment of ecotoxicants effect in the whole animal through the use of high density microarray screening of zebrafish embryos in combination with large-scale biological replication and a balanced ANOVA statistical design.

Zebrafish embryos were semi-statically exposed at EC₁₀ and 50% EC₁₀ over 72 h, and the resulting effects on gene expression determined. Cluster and statistical analyses have been used to identify common and differentiated gene expression patterns, and sophisticated system-wide pattern searching algorithms to identify biological pathways and processes affected.

The resulting high resolution datasets allow expression patterns to be used as fingerprints to identify ecotoxicants, for high-throughput screening of chemicals, or for the investigation of modes of toxicity through association with altered regulation of response pathways. Initial testing with four chemicals has shown that this methodology is highly amenable to all three uses. The gene expression profiles for the different toxicants were distinctive, and analysis using Gene Ontology profiling, Reactome or network-based packages provided a high level of detail of affected processes. Ongoing research aims to substantially increase the predictive power of the technique, and to extend the project to use cell cultures.

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A4.12

16:05 Sunday 28th June 2009

In vitro techniques and their application to nanoparticles

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Engineered nanomaterials have unusual physico-chemical properties (review, Handy et al., 2008, *Ecotoxicology*, 17, 287–314), and it is unclear whether cell culture or organ perfusion methods will work well with these materials. The aim is to review these methods for their application to nanoparticles (NPs). We find that there are no fundamental reasons why these techniques should not work with NPs, but there are technical problems to resolve. Dosing of the

external medium (e.g., physiological saline, culture media) requires a dispersion of NPs, and features of the media such as ionic strength, will alter NP dispersion. Good experimental design with controls, solvent controls, and a range of doses remains possible. One concern for perfused organ preparations is that NPs may aggregate in blood vessels or capillaries, impacting on organ viability; but this can also be interpreted as a feature of toxicity. For cell cultures, the complexity of the media is a source of uncertainty, and it is not yet clear how the various solutes and supplements (e.g., bovine serum albumin or antibiotics) will interact with different types of NPs. There are some concerns that NPs might interfere with cell adhesion processes. The end points used for *in vitro* studies also require consideration, and additional checks in methodology should be made for each new material. For example, ensuring that the optical properties of NPs do not interfere with colorimetric assays, or give false positives/negatives in such assays.

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A4.13

16:25 Sunday 28th June

Agglomeration of tungsten carbide nanoparticles in exposure medium does not prevent uptake and toxicity toward a rainbow trout gill cell line

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One phenomenon widely described for nanoparticles in suspension is their tendency to agglomerate. Agglomeration depends on the type of particles and the composition of the aqueous suspension in which they are kept. An increasing number of *in vivo* and *in vitro* studies on the toxicology of nanoparticles have been conducted with both mammalian and non-mammalian species; yet, it remains unknown if nanoparticle uptake and toxicity is influenced by agglomeration. This is because comparative test systems, where uptake and toxicity can be studied with or without agglomeration, have not yet been proposed. We therefore decided to employ a fish cell line that is amenable to exposure in media with differing complexity. This cell line, the rainbow trout gill cell line, RTgill-W1, was exposed to two well characterised nanoparticle types, tungsten carbide (WC) and tungsten carbide cobalt (WC-Co). We demonstrate that WC and WC-Co nanoparticles are able to enter the cells regardless of whether cells are exposed in media that prevent (serum present) or favour (serum absent) the formation of particle agglomerates. Short-term exposure of the cells led to significant cytotoxicity at the highest nominal particle concentrations irrespective of the particle type or medium composition. In contrast, long-term exposures led to preferential toxicity in the simplest medium and an enhanced toxicity by the cobalt-containing WC particles. However, under no condition could the ionic cobalt alone explain the

toxicity of the WC-Co particles, suggesting that the combination of metallic Co and WC is the cause of the increased particle toxicity of WC-Co.

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A4.14

Poster Session – Monday 29th June 2009

Neutral red uptake assay with coelomocytes of *Eisenia fetida* as an effective and reliable screening method for soil health assessment

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The aim of the present study was to develop a screening procedure for soil toxicity testing based on the Neutral Red Uptake (NRU) assay in extruded coelomocytes of the earthworm *Eisenia fetida* exposed *in vivo* to inorganic and organic contaminants. One variable to take into account in order to optimize the cost-effectiveness of the protocol is to determine the shortest effective exposure time needed to observe responses that may be indicative of alterations in soil health status. Thus, short term (up to 3 days) and short to intermediate term (from 3 to 28 days) experiments were carried out. In both experiments copper, zinc, lead and kerosene were selected as model contaminants. For all the contaminants tested a significant decrease in the activity of coelomocytes was measured at short periods of time (up to 3 days). Longer exposures produced an increase in the NR uptake probably due to toxic or adaptive effect occurring beyond 3d of exposure. In conclusion, the combination of one experimental design of 3 days of duration exposing stocked *Eisenia* to a set of selected contaminants together with an implemented, rapid and sensitive NRU protocol is a cost-effective tool for the assessment of the general status of soil contaminated by organic and metallic contaminants. Moreover, the simplicity, rapid performance and the possibility to deal with a high amount of different soils (high-throughput) result relevant for decision making in soil management.

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A4.15

Poster Session – Monday 29th June 2009

Isolated strains of *Cylindrospermopsis raciborskii* from Lake Balaton (Hungary) produce anatoxin-a like neurotoxins

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The membrane effects of the partially purified extracts (from 100 mg of freeze-dried material) of the cyanobacteria *Cylindrospermopsis raciborskii* ACT 9502, ACT 9504, ACT 9505 isolated from Lake Balaton, Hungary were characterized on identified central neurons of

the pond snail *Lymnaea stagnalis*. The toxic pattern of solid phase extracted fractions was screened against the partially purified anatoxins produced by the positive reference strain *Oscillatoria formosa* (PCC 6506).

The purified fraction of PCC 6506 strain (1 mg dry weight/ml) evoked strong intracellular (either depolarizing or hyperpolarizing) membrane responses on the identified neurons (buccal B1, B4 neurons, the pedal RPeD1 neuron), similar to the locally applied acetylcholine, iontophoretically injected near the surface of the cell body. PCC 6506 applied by perfusion in lower (0.2–0.5 mg/ml) concentration reversibly blocked this acetylcholine responses, suggesting inhibition of the cholinergic receptors. The similar reversible, dose-dependent inhibition of acetylcholine responses was recorded in the presence of the authentic neurotoxin anatoxin-a (1–10 μ M) applied by perfusion. The extracts prepared from *Cylindrospermopsis raciborskii* (ACT 9502, ACT 9505) had similar cholinergic agonist effect as well as blocking the acetylcholine responses suggesting an anatoxin-a like component produced by the strains isolated from Lake Balaton.

Liquid chromatography-mass spectrometry (LC-MS) confirmed the presence of the cyanotoxins anatoxin-a and homoanatoxin-a in relevant amounts in the PCC 6506 strain, and in trace amounts in the cyanobacterial mats of *Cylindrospermopsis raciborskii* strains isolated from Lake Balaton.

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A4.16

Poster Session – Monday 29th June 2009

A reporter gene assay to test for pollutant activation of fish peroxisome proliferator-activated receptors

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A growing number of chemical contaminants are known to disrupt endocrine signalling in fish by interacting with receptors of sex steroid hormones. Other members of the nuclear receptor gene family, such as peroxisome proliferator-activated receptors (PPARs) and corticosteroid receptors (CRs), also have important endocrine functions in fish, regulating energy homeostasis by controlling the transcription of genes involved in lipid, protein and glucose metabolism. Relatively little is known about their interaction with chemical contaminants present in the aquatic environment.

Here, we report a high-throughput luciferase assay designed to test pollutants for interaction with PPAR signalling in flounder. The ligand-binding domains of flounder PPARs were expressed as fusion proteins with the DNA-binding domain of the yeast GAL4 transcriptional regulator in an Atlantic salmon (AS) cell line. Ligand-dependent transactivation by the receptors was monitored using a luciferase reporter gene fused to a GAL4 response element. This system minimizes background “noise” from endogenous receptors, which are unable to bind to the GAL4 response element. In our assay, known PPAR agonists produced the expected responses, and several environmental chemical contaminants induced transactivation of flounder PPAR α .

Similar experiments will be used to test for interactions between chemical contaminants and CRs. Currently partial cDNA sequences for three CRs, coding for two glucocorticoid and one mineralocorticoid receptor, have been isolated from flounder liver. Phylogenetic analysis

shows that these flounder receptors have one-to-one orthologues in most available teleost genomes.

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A4.17

Poster Session – Monday 29th June 2009

Organ perfusion studies in fish; kidneys and gills

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This presentation will discuss the potential usefulness of perfused fish organ techniques. In contrast to mammals, poikilothermic animals are convenient for organ perfusion studies which can be carried out at temperatures where their relatively low oxygen consumption means that saline solutions can carry enough oxygen in physical solution to meet tissue requirements. Isolated perfused gill arches have proved useful for study of vascular effects of hormones and the way they are affected by pollutants. They are less suitable for ion transport studies than flat tissues such as opercular epithelia because of their complicated vascular system.

As we evolved from fish-like ancestors our kidneys have features in common with those of fish, study of which made important contributions to the early development of renal physiology. The differences are equally interesting, for example glomerular filtration rates which depend on perfusion pressure and vary by a process of glomerular recruitment. Differences in anatomy include the presence of a renal portal system, allowing the tubules to be perfused independently of the glomeruli. In aglomerular fish the arterial supply has been lost, together with the glomeruli, and urine is produced by tubular secretion from the portal circulation. The compact anatomy of the two separate kidneys of the Angler Fish, *Lophius piscatorius* is very convenient for their rapid removal from the fish and perfusion via the single branch of the renal portal vein to each kidney to study factors affecting tubular function.

Perfused trout kidney data, including unpublished results on peptide hormone actions, will be presented.

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A4.18

Poster Session – Monday 29th June 2009

Exploring the role of dosing procedure and chemical properties in *in vitro* assays using a fish gill cell line

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The bioavailability of chemicals within cell cultures influences the predictive ability of *in vitro* toxicity tests. Potentially results can be influenced by the physico-chemical properties of the putative toxicants, the exposure vessel and medium, and the manner in which cultures are dosed. For example, lipophilicity (described by logP) drives sorption to exposure medium components, like serum proteins, and to the walls of exposure vessels, like micro-wells. Further, volatility (described by logHLC) determines losses due to evaporation during handling or exposure. Support for these ideas comes from a recent comparison of data bases of mammalian cell line and fish acute toxicity where we indeed were able to explain a large part of the differences between *in vivo* and *in vitro* by the chemicals' logP and logHLC. Based on these findings we systematically studied the influence of physico-chemical properties of chemicals and of handling procedures on cell viability within the first few hours of exposure and 24 h later. A rainbow trout gill cell line, RTgill-W1, was used because the cells survive for days in a simple basal medium and without serum, which presumably reduces binding to medium components, and can be exposed at ambient temperature, which aids in clarifying evaporation effects. We will present our analysis on the sensitivity of the outcome of cell viability based on factors such as logP, logHLC, dosing method, solvent used, and provide recommendations for improved test set-ups that are applicable to both fish and mammalian cell lines.

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An integrated approach to determine relative sensitivity of mammalian and fish cells: biochemical, cytogenetic and cellular studies

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