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A2–CELLULAR MECHANISMS OF TOXICITY

Organised by Richard Handy and Nic Bury for the SEB Osmoregulation Group

A2.1 Metal physiology and biochemistry in fish cells: from toxicity to protection by zinc

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Zinc can be a toxicant to fish, but has also well recognised biological functions. We investigated the ability of zinc to mobilize defense against reactive oxygen species (ROS) using a primary culture of rainbow trout gill cells. Exposure of cells to zinc, H_2O_2, sodium nitroprusside (SNP) or 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) resulted in upregulation of mRNA for metallothioneins (MT-A, MT-B), glutathione S-transferase (GST) and glucose 6-phosphate dehydrogenase (G6PD). The stimulatory effects of zinc or H_2O_2 on MTA, MTB, GST and G6PD mRNA levels could be blocked by addition of the membrane permeable zinc chelator, N,N,N′,N′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), suggesting that ROS-induced upregulation of these genes is zinc-dependent. Pretreatment with zinc protected the cells from subsequent cell damage and apoptosis, as assessed by lactate dehydrogenase leakage, mitochondrial dehydrogenase activity (MTT assay), caspase-3 activity, and DNA fragmentation. In contrast, when gill cells were coincubated with zinc and H_2O_2 at the same time, H_2O_2 toxicity was higher than after treatment with H_2O_2 alone. It is concluded that zinc had a direct pro-oxidant effect when administered together with H_2O_2, but that pretreatment of zinc inhibited cytotoxicity and apoptosis through an indirect antioxidant action. We propose that the antioxidant action is manifested through zinc-dependent expression of several genes encoding antioxidant proteins. Furthermore, the apparent zinc-dependency of ROS-induced expression of antioxidant genes suggests that zinc might act as a physiological signal to mediate the response to oxidative stress.

A2.2 Essential metal ion pathways and regulatory networks

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The role essential metal ions play within protein structure and enzyme function is well established. Furthermore, the maintenance of the homeostatic balance between toxicity and depravation of these inorganic elements is critical to maintain the integrity of cellular function. Individual components responsible for uptake, intercellular sequestration/compartmentalisation and efflux together with a small number of transcription factors responsible for controlling metal dependant pathways have been identified and characterised in isolation. Restricted studies have previously described pathways mediating delivery of Cu to individual cellular components and a regulatory network controlling the translation of components of the Fe uptake system. The present paper presents how the new techniques in systems biology (bioinformatics, genomics, and transcriptomics), coupled with comparative genomics, presents an opportunity to identify a far wider spectrum of pathways and regulatory networks underlying inorganic biochemistry. This data may be used to create more complex models relating to inorganic homeostasis and the potential impact of disruption of these systems, caused through genetic mutation, environmental excess/deficiency and antagonistic effects of toxic metal ions (such as cadmium and mercury), to perturb cellular metabolism. These approaches present an opportunity for a paradigm shift in the way we approach metal ion biology, from studying individual component to the description of their full cellular context.

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A2.3 Effects of copper on hepatic gene expression in *Fundulus heteroclitus*

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Due to its redox properties and essentiality, tight regulation of copper at the whole animal, organ, and cellular level has evolved. The liver is the main homeostatic organ in vertebrate copper metabolism. Because of this, we are analyzing the gene expression changes in the liver of *Fundulus heteroclitus* exposed to different concentrations of copper in several salinities. Both copper accumulation and other physiological responses were greatly influenced by salinity. Therefore, hepatic gene expression patterns are expected to differ. The gene expression patterns for 6,144 hepatic genes currently are being quantified in individual fish using at least two slides containing triplicate arrays. Samples for microarray analysis were selected based on the concentrations of copper accumulated in the liver as well as other measured physiological responses of the fish to optimize our chances of investigating primarily copper responsive genes. Exposed individuals ($n=3–4$) will be compared to appropriate simultaneous controls. The statistical analysis and the experimental design are based on the method described by Oleksiak, Churchill, and Crawford (*Nature Genetics*, 2002, 32: 261–266). The predicted changes in gene expression patterns will be interpreted in light of the physiological data obtained from the very same fish to hopefully identify copper related gene expression changes.

A2.4 The interaction of iron with the gills of freshwater teleost fish – A physiological, biochemical and molecular perspective

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Iron is essential for life, but in excess is extremely toxic. Iron present in water is presumed to be unavailable to fish due to the formation of insoluble hydrous iron oxides or being bound to organic material. However, results show that iron enters freshwater rainbow trout via the gill. Various iron chelators (log K_1 $Fe^{3+} > 10.2$) significantly reduce, but not abolish iron uptake, suggesting that chelated iron is bioavailable. A possible explanation for this bioavailability is the cycling of bioavailable ferrous iron (Fe^{2+}). In the absence of the chelators up to 5% of the iron is present as Fe^{2+}. However, in the presence of the iron chelator desferroxiamine (DFO) no Fe^{2+} was measured and all iron remains bound to DFO. Preliminary studies have shown that the gills may secrete large quantities of ascorbate, and we hypothesis that the gill generates a reducing microclimate capable of converting Fe^{3+} to Fe^{2+}. The likely candidates for Fe^{2+} import are members of the solute carrier 11a (Slc 11a) family of transport proteins, [previously known as divalent metal transporters (DMT)]. Using a *Xenopus* oocyte expression system, the genes in rainbow trout that are homologous to the mammalian Slc 11a genes import iron. This import is pH sensitive indicating that they act as Fe^{2+}/H^{+} symporters. Competition studies with other divalent metals shows that Cd^{2+} prevents Fe^{2+} entry *in vivo* and *in vitro*, signifying that this extremely toxic metal may enter fish via this transport process.

A2.5 Heavy metal toxicity in neuronal and glial cell lines

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The effects of the heavy metals mercury, lead, zinc and cadmium on N2a (mouse neuroblastoma) and C6 (rat glial) cell lines were investigated. These cell lines were induced to differentiate. Cell morphology studies and MTT reduction assays were then used to determine the relative sensitivities of these cell lines to the heavy metals. For the cell morphology studies cells were fixed at various times after addition of the heavy metal, and the number of axons (N2a) or cell processes (C6) were counted. In healthy cells the dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is reduced to MTT formazan (1-[4,5-dimethylthiazol-2-yl]-2,5-diphenylformazan). This results in a change in colour of the MTT dye from yellow to purple and is taken as an index of metabolic activity. For both cell morphology and MTT reduction assays lead and zinc were markedly less toxic than either cadmium or mercury. Further screening studies confirmed that organic mercury is more toxic than inorganic mercury. Cell extracts of mercury exposed N2a and C6 cell lines were then subject to SDS PAGE and Western blotting. These blots were then probed with antibodies that recognise neurofilaments (N52), phosphorylated neurofilaments (RT97), total alpha tubulin (B512) or tyrosinated alpha tubulin (T1A2) for the N2a cell line, and with the B512 and T1A2 antibodies for the C6 cell line. Preliminary results indicate that sublethal mercury concentrations alter the levels of neurofilaments and tubulin detected by the antibodies and that organic mercury is more potent than inorganic mercury.

A2.6 Mechanisms of dietary mercury uptake and toxicity: fish versus mammalian models

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The health risk associated with mercury exposure has historically led to research on mammalian models of mercury toxicity. However, genomic research has highlighted the value of gene hopping approaches using fish models. This paper determines whether fish are a better model than rats for exploring the mechanisms and aetiology of dietary mercury toxicity. In the rat jejunum (e.g. Foulkes & Bergman, TAP, 120, 89–95), exposure of gut sacs to 20 μmol/l $HgCl_2$ results in fast (chelator-sensitive) and slow components of uptake (40 and 24 nmol/g/min at 37 °C); representing surface bound and internalized mercury pools. Both slow and fast components are temperature sensitive, and Hg^{2+} uptake into the gut tissue increases by 62% on removal of Cl_o but is not DIDS- sensitive. The intrinsic passive permeability of the rat gut to mercury declines after weaning. Caco-2 cells show a similar passive component. The K_m for inorganic Hg uptake is about 10 μmol/l for $HgCl_2$ in mammalian intestine, and similar in fish, but even accounting for temperature differences, maximum uptake rates for inorganic Hg uptake in fish intestine are 10 fold lower. The pathway for luminal inorganic Hg to enter the gut cells is amiloride-sensitive in fish, and in both fish and mammals luminal Ca stimulates Hg uptake. The target organs for dietary Hg compounds are similar in mammals and fish, and include the brain, gut, liver and kidney. Oxidative injury to tissues and similar brain pathologies (vacuolation, astrocyte proliferation, foci of necrosis e.g. Berntssen et al., Aq. Tox. 65, 55–72) are implicated in dietary Hg toxicity in salmonids and mammals. The pathology suggests dietary mercury enters the brain via the spinal cord in both fish and mammals.

A2.7 Temperature and cadmium synergistically affect mitochondrial function in a marine poikilotherm, *Crassostrea virginica*

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Marine intertidal mollusks such as oysters, are exposed to multiple stressors in estuaries, including temperature and trace metals, which may interactively affect their physiology. In order to understand combined effects of cadmium and elevated temperature on mitochondrial bioenergetics of marine mollusks, respiration rates and mitochondrial volume changes were studied in response to different cadmium levels (0–1000 μM) and temperatures (15, 25 and 30 °C) in isolated mitochondria from the eastern oyster *Crassostrea virginica* acclimated at 15 °C. It was found that both cadmium and temperature significantly affect mitochondrial function in oysters. Exposure of oyster mitochondria to 35 °C resulted in a decreased respiratory control and phosphorylation efficiency (P/O ratio) compared to the acclimation temperature (15 °C), while intermediate temperature (25 °C) had no effect. Cadmium exposure did not lead to a significant volume change in oyster mitochondria *in vitro*. Low levels of cadmium (1–5 μM) stimulated the rate of proton leak in oyster mitochondria, while not affecting ADP-stimulated state 3 respiration. In contrast, higher cadmium levels (10–50 μM) had little or no effect on proton leak, but significantly inhibited state 3 respiration by 40–80% of the control rates. Elevated temperature increased sensitivity of oyster mitochondria to cadmium leading to an early inhibition of ADP-stimulated respiration and an onset of complete mitochondrial uncoupling at progressively lower cadmium concentrations with increasing temperature. Enhancement of cadmium effects by elevated temperatures suggests that oyster populations subjected to elevated temperatures due to seasonal warming or global climate change may become more susceptible to trace metal pollution and *vice versa*.

A2. 8 Physiological responses of the freshwater crab, *Potamonautes warreni* Calman to waterborne cadmium and microbial gill infestations

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Adult freshwater crabs (*Potamonautes warreni*), harbouring heavy microbial gill infestations and acclimated to Cd^{2+} (0.009 mg/L) and NH_4^+ (0.12 mg/L) in their natural habitat in the Mooi River, at Noordbrug, North-west Province, South Africa, were experimentally exposed to 0.2 mg Cd^{2+} /L for 7, 14 and 21 days at 24 °C. In acclimated infested crabs, initial changes in heart rate, NH_4^+–N, O:N, osmotic pressure in the haemolymph became stabilised after 14 days, whereas after 21 days the mean growth rate and Mo_2 declined and pH increased significantly. The microbial gill fauna was eliminated after 7 days, accompanied by a decrease in MO_2, heart rate and O:N ratio, and an increase in NH_4^+–N and osmotic pressure in the haemolymph. After 14 days Cd particles were deposited on the tips of microvilli of the gill epithelial cells, in which mitochon-

drial swelling and dissociation of cristae occurred. The heart rate, MO2, O:N ratio and retention in NH_3/NH_4^+ increased compared to a decrease in the mean growth rate and NH_4^+ excretion. After 21 days a 2-fold increase in growth rate and a decrease in NH_3/NH_4^+ retention were accompanied by a rise in the mean osmotic pressure and pH in the haemolymph and a 3-fold increase in the O:N ratio, which was significantly inversely correlated with NH_4^+–N ($r=-0.98$; $P=0.0004$). Integrated cellular and physiological responses by *P. warreni* and the mode of action and interaction of both stressors, ie cadmium toxicity and microbial infestations, on the health status of *P. warreni* are discussed.

A2.9 Mechanisms of endocrine toxicity: Is there a causal association between genotoxicity and the imposex effect?

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It is well established that some hormones can act as cellular proliferators and could therefore serve as cancer promoters or epigenetic carcinogens. In addition, growth-stimulating effects may promote cancer by increasing the proliferation rate of cells that have acquired a mutation either spontaneously or following exposure to chemical pollutants. New evidence indicates that hormonal metabolites may directly induce damage to genetic material, raising the possibility that pollutant-induced endocrine disruption might not occur in isolation, but might be just one of a suite of toxicological effects. In order to explore this hypothesis, we studied two phylogenetically diverse organisms, the trout Rutilus rutilus and the dogwhelk, Nucella lapillus, exhibiting recognisable alterations in their endocrine systems, for evidence of genetic damage. In mesocosm studies, R. rutilus exposed to sewage effluent sufficient to induce the induction of vitellogenin showed a concomitant increase in genotoxic damage (micronucleus formation). Similar results were obtained from a field study of N. lapillus exposed to the antifouling agent tributyltin (TBT), indicating a correlation ($R^2=39.57$, $p=0.0001$) between DNA damage (micronucleus assay, Comet assay) and incidence of imposex (masculinisation of females). TBT is known to induce apoptosis in mammalian lymphocytes and neurones, but has not previously been shown to induce genotoxicity. The possible mechanisms of action of these effects, either through direct damage to DNA, induction of apoptosis, or modulation of signal transduction pathways that normally regulate cell cycle repair checkpoint functions, is discussed in relation to pollutant-induced disruption of endocrine processes in different organisms.

A2.10 Apoptosis induced by cell shrinkage

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Change in cell volume is a signal used in several physiological mechanisms. Thus cell proliferation is augmented by cell swelling and cell shrinkage is an integral part of the event leading to programmed cell death (apoptosis). We find that hypertonic cell shrinkage *per se* can initiate the apoptotic process in NIH3T3 fibroblasts. Longer lasting cells shrinkage results in activation of the stress-activated kinases, c-Jun-N- terminal kinase (JNK) and p38 mitogen activated protein kinase (p38MAPK) after about 30 and 5 minutes respectively, but not in extracellular regulated kinases (Erks), that instead are activated by cell swelling. Shrinkage activation of stress kinases is followed by phosphorylation on serine in position 15 (S15) of the multifunctional transcription factor p53 after about 30 minutes and by activation of the apoptotic enzyme caspase-3 after about 112 hour. An increased permeability to cations and to organic osmolytes is seen after 1 hour. The process is dependent on the small G protein Rac. Immunofluorescense microscopy analysis demonstrates that shrinkage induced phosphorylation of p38 and p53 in hypertonic medium is associated with a significant increase in the nuclear localization of p38, phospho-p38, p53 and phospho-p53. Based on these findings we conclude that hypertonicity-induced cell shrinkage is associated with apoptosis, involving the activation of Rac, p38, JNK1 and p53. We are presently examining the expression of a number of down-stream effectors of p53 known to play key roles in the apoptotic process to obtain further information on the signaling cascade during shrinkage-induced apoptosis.

A2.11 Stress and volume regulation in red cells

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Many animal cell types are able to counteract cell volume disturbances by activation of transport mechanisms that redistribute solutes and osmotically drawn water across the cell membrane. Thus, upon osmotic swelling, systems like the K,Cl-cotransporter (KCC) extrude ions and osmotically obliged water from the cell, leading to a regulatory volume decrease (RVD). Similarly, upon osmotic shrinkage, systems like Na, K, 2 Cl-cotransport (NKCC) or Na, H-exchange (NHE) working in parallel with the anion exchanger, lead to a net uptake of ions

and water into the cell, resulting in a regulatory volume increase (RVI). However, several studies have shown that various stressors can activate some of these volume regulatory transport system even under isotonic conditions, often leading to large deviations of cell volume from the unstressed condition. In this overview the effects of increased plasma catecholamine levels, high oxygen tensions and changes in pH on cell volume and KCC, NKCC and NHE activity are assessed in isotonic red blood cells of several vertebrate species. The analysis shows the importance of distinguishing between stress-activated volume regulatory transporters as part of the cause of, or part of the remedy against, a certain stressor.

A2.12 Mechanisms of stress resistance

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Dysfunction of cellular lysosomes is a recognised mechanism of cell injury induced by many stressors including pollutants. Recent investigations on mammalian and molluscan cells indicate that prior induction of lysosomal autophagy protects cells against oxidative injury that would otherwise lead to programmed cell death. Physiological function emerges from the interactions between cellular proteins: pollutant-induced damage to cellular proteins will, therefore, impact on integrated physiological function. Consequently, increased removal of proteins damaged by reactive oxygen species (ROS) and adduct formation with reactive ligands, by means of improved autophagic proteolysis, will probably result in improved cellular 'housekeeping' and help to maintain function during stress. Both mooluscs and mammals show an age-related decline in lysosomal function; however, dietary restriction (DR) is believed to improve autophagic/lysosomal capacity and this confers anti-ageing protection. Autophagy and lysosomal function are highly conserved evolutionarily; and it is likely that this ancient system has remained largely unchanged because it conferred anti-ageing and stress resistant properties. Starvation of blue mussels exposed to copper and phenanthrene reduced the severity of lysosomal damage and there was full recovery of lysosomal integrity after 12 days in clean seawater. Autophagy is triggered by DR, salinity increase and hypoxia in mussels. Since these conditions often prevail in estuarine environments, the repeated triggering of autophagy may be a significant contributory factor to stress tolerance in mussels and other intertidal animals. The environmental significance of this lysosomal-autophagic protection system remains to be fully assessed, but we can speculate that it may play a protective role in the survival of animals chronically exposed to stress and pollution.

A2.13 Experimentally-induced feminisation of freshwater mussels (*Elliptio Complanata*) after long-term exposure to a municipal effluent (Saint-Lawrence River, Quebec, Canada)

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As other oviparous organisms, bivalves produce vitellin-like (egg yolk) proteins (Vn) presumably under estrogen receptor mediation. After confirming that bivalve Vn is a Zn- and Ca- containing glycolipophosphoprotein similar in constitution to yolk protein found in fish, we used an alkali labile phosphate (ALP) method to indirectly measure Vn expression in field-exposed animals. The ALP method was then employed in studies to identify areas having the potential to affect the estrogenicity of bivalves. In the present study, we examined sex ratio effects on the freshwater mussel *Elliptio complanata* after a long-term (one-year) exposure to a municipal effluent plume in the St. Lawrence River (Quebec, Canada). *Elliptio complanata* mussels were exposed in specially-designed benthic cages firmly attached to the bottom with spikes and placed upstream (2 km) and downstream of the municipal effluent plume (7 and 10 km) in June 2001. Cages were retrieved in June 2002 and mussels were measured for multiple growth metrics, vitellogenin-like proteins and sex ratio. Results showed that mussels from the two downstream sites had higher condition factor and gonado-somatic index when compared with those from the upstream site. They also displayed significantly more vitellogenin-related proteins in their gonads. The proportion of females was significantly higher downstream (62 and 66%) than upstream (41%). This high proportion of females is not usually observed in natural populations of *Elliptio complanata* and suggests sex reversal was induced in this field study. The most likely explanation is exposure to estrogenic compounds associated with the municipal effluent.

A2.14 Mechanisms of endocrine disruption – A molecular approach

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The endocrine system facilitates communication between cells to coordinate multiple physiological processes and maintain homeostasis. It is now well established that there are a wide variety of chemicals that can mimic and/or interfere with endocrine system in the body and in turn lead to alterations in growth, development and/or reproduction. Disruptive effects of endo-

crine disrupting chemicals (EDCs) have been found in a range of wildlife populations, including birds, amphibians, mammals and fish. EDCs are also thought to be implicated in the reduction of human sperm counts and the increase in some hormone dependent (reproductive) cancers.
EDCs can potentially affect a wide range of endocrine targets in the body, but most studies to date have focused on effects mediated via the androgen, oestrogen and thyroid pathways only. Our work on endocrine disruption has focused on impacts of EDCs on fish reproductive health and we have cloned a wide range of genes that play central roles in reproduction (e.g. oestrogen receptors, various enzymes involved with sex hormone biosynthesis and gonadotrophins) with a view to developing targeted approaches for unravelling the mechanisms of sexual disruption. Some chemicals, however, can act at multiple targets to disrupt physiological function (e.g. nonylphenol). Development of more comprehensive molecular approaches (e.g. fish gene arrays) are therefore required if we are to more fully appreciate the interactions of chemicals with the endocrine system and identify pathways and mechanisms of endocrine disruption. Gene array approaches for this purpose will be discussed.

A2.15 In vitro toxicity of cadmium and copper to snail hepatopancreas cells

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The toxic effects of cadium and copper on cellular metabolism and cell morphology were investigated in isolated hepatopancreas cells from Helix pomatia. Cell viability was unaffected during 1 hour of incubation with 100 μM Cd, but was significantly reduced with 100 μM Cu and 500 μM Cd. The adverse effect of Cd on cell viability was not observed in cells isolated from Cd pre-treated snails. Oxygen consumption was unaltered by 100 μM Cu, whereas 500 μM Cd caused a significant reduction in both aerobic ATP production and in Na^+/K^+-ATPase activity, as estimated from the ouabain-sensitive rate of oxygen consumption. Hepatopancreas cells showed enhanced formation of reactive oxygen species when exposed to Cu, but not in the presence of Cd. Morphologically, we observed an increase in cell volume of Cd-exposed cells, while cell membrane bleb formation was induced by both metals. The latter result was underlined by visualising the F-actin filamentous network of the cells, which showed distinct staining within the blebs of the cell membrane. Overall, our results indicate that Cd and Cu are acutely toxic for hepatopancreas cells from the Roman snail, with Cu being more toxic than Cd. While Cd-induced toxicity seems to occur via effects on energetic metabolism, Cu appears to exert its toxic effects at the cell membrane and involves the formation of recative oxygen species.
Supported by the Austrian Science Foundation, project no. P-14593-MOB and F. Antorchas, UBA, CONICET, ANPCYT (01-11017).

A2. 16 Heart rate, hatching success and mortality in early development in zebrafish (*Danio rerio*) larvae exposed to bioactive extracts of the freshwater cyanobacterium, *Microcystis aeruginosa,* strain PCC 7813

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Cyanobacteria, or blue-green algae, are ancient photosynthetic organisms that grow in marine, brackish and fresh waters. During blooms, *Microcystis* species may produce a variety cyanobacterial toxins such as microcystins. Ingestion of these hepatotoxins in higher vertebrates causes variety of symptoms including damage to the liver. Soluble glutathione S-transferase (sGST) has been shown to be involved in the initial step of microcystin detoxication. Most toxicity studies have shown that exposure to microcystins has sub-acute effects on freshwater fish at environmentally-relevant concentrations. Fish kills are sometimes associated with cyanobacterial blooms although mortalities are not clearly attributable to the cyanobacteria or their known toxins. Some compound(s) other than microcystins may be responsible for stimulating the acute stress response. The zebrafish has become a model of vertebrate development and disease and also a new animal model for cardiac research. It is easily obtainable, inexpensive, and provides a practicable and highly sensitive bioassay for the assessment of toxic materials. The objective of this study was to assess the effect of bioactive extracts of *Microcystis* PCC 7813 on developmental biology of zebrafish, examining hatching success, morphology, larval heart rate and mortality. Hatching success and mortality were unaffected apart from at high concentrations (2000 μg microcystin-LR equivalent L^{-1}). After 24 and 48 hours exposure, heart rate increased with increasing concentration of treatments. In general no developmental deformities were observed.

A2.17 Using Biochemical Biomarkers to differentiate modes of Arsenic and Copper Toxicity: $Na^{+}K^{+}$-ATPase, TBARS and Metallothionein Induction in the Zebra Mussel, *Dreissena polymorpha* and Freshwater isopod, *Asellus aquaticus*

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The application of general health biomarkers within environmental studies may overlook toxicity attributable to the various intracellular mechanisms of different heavy metals. Experiments focus on the sublethal toxicity of copper and arsenic to organisms occupying different trophic levels. The freshwater species *Dreissena polymorpha* and *Asellus aquaticus* were exposed to copper (100 $\mu g\ l^{-1}$) or trivalent arsenic (140 $\mu g\ l^{-1}$) over 7 days. Sub-lethal effects of each metal was investigated by measuring metallothionein (MT) induction, thiobarbituric acid reactive substances (TBARS) and whole body $Na^{+}K^{+}$-ATPase activity. Both invertebrate organisms showed increased levels of MT during the Cu exposure and transient changes in lipid peroxidation as indicated by TBARS. *Asellus* exposed to As displayed an initial rise in TBARS level which decreased toward background levels over the remaining 4 days, and was complemented by a significant ($P<0.05$) 37% elevation of MT (to 503.9 ug MT mg protein^{-1}). *Dreissena* exposed to As showed no significant change in TBARS levels, however, MT was noted to be 12% higher than untreated samples after day 3 (480.9 ug MT mg protein^{-1}) albeit not to any significant level, dropping at day 7 (454.5 ug MT mg protein^{-1}). No inhibition of $Na^{+}K^{+}$-ATPase activities was noted over 7 days for exposed organisms, and baseline values noted here, *A. aquaticus* 1.1 μmol Pi mg $^{-1}$ hr^{-1} and *D. polymorpha* 0.38 μmol Pi mg $^{-1}$ hr^{-1}, are thought to be the first for these species. In conclusion, the application of a suite of invertebrate heavy metal biomarkers gives clarity and sensitivity to an environmental study.

A2.18 Role of mitochondria in cadmium-induced apoptosis in hemocytes of the eastern oyster *Crassostrea virginica*

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Apoptosis plays an important role in the immune system and can be induced by a variety of external and internal stimuli. Cadmium is a well known environmental cytotoxin that induces apoptosis in eukaryotic cells. In the current study we have investigated the effects of cadmium on isolated oyster hemocytes, which are the main cellular immune defense in mollusks. Cadmium exposure induced apoptosis in a dose-dependent manner in the range of 10–100 μM, as indicated by the translocation of phosphatidylserine to the outer leaflet of the plasma membrane. At higher concentrations (200–1000 μM) there was no further increase in apoptosis but a significant increase in the level of necrosis. Cadmium exposure in the apoptotic range caused a significant decrease in intracellular ATP levels, indicating a severe disturbance of energy metabolism. However, there was no decrease in the mitochondrial membrane potential, suggesting that the opening of the mitochondrial transition pore (MTP) is not involved in the induction of apoptosis in oyster hemocytes. These results contrast the earlier findings in vertebrates in which cadmium-induced apoptosis involved opening of the MTP and mitochondrial depolarization and suggest that trace-metal induced apoptosis may proceed by a different pathway in marine mollusks. Indeed, cadmium exposure actually caused a significant hyperpolarization of mitochondrial membrane by 10–15%, demonstrating that the observed decrease in ATP production is not due to the loss of the mitochondrial protonmotive force but more likely due to inhibition of the F_0, F_1-ATP synthase and/or of mitochondrial ADP/ATP transport.

A2.19 Metabolic responses associated with ethinylestradiol- or methyltestosterone-exposure in the pregnant eelpout (*Zoarces viviparus*)

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A significant dose-related increase in the level of the yolk-precursor protein vitellogenin and calcium was observed in plasma of pregnant eelpouts (*Zoarces viviparus,* L) exposed to EE2 and E2 in the ambient seawater. Increased protein synthesis activity was observed concomitantly in the liver of the estrogen-exposed fish by PCR. A significant increase was observed in the

erythrocyte nucleoside triphosphate (NTP) concentration in the blood of female fish exposed to the high dose of MT (500 ng/L) during early pregnancy. No other significant effects were observed in the different hematological parameters, which were measured as possible indicators of xenobiotic effects. Hepatic activity of different enzymes related to protein and glucose metabolism or indicative of oxidative stress response was investigated and dose-related changes were observed. A different pattern in the hepatic activity of the measured enzymes including the glycolytic enzyme pyruvate kinase and glucose-6-phosphate dehydrogenase (G6PDH) was observed when expressed in hepatic units, specific activity or normalized to the hepatosomatic index. G6PDH is the major provider of NADPH, which is important as reducing power for detoxification pathways and is thus an interesting enzyme in relation to xenobiotic exposure.

A2.20 Toxicity and sub-cellular effects of fish farm chemotherapeutants on non-target marine crustaceans

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In some Scottish coastal waters, the salmon aquaculture industry relies on the application of chemotherapeutants for effective treatment of sea lice (*Lepeophtheirus salmonis*) infestations. The most commonly applied treatments are cypermethrin (bath treatment applied as Excis®) and emamectin benzoate (in-feed treatment applied as Slice®).

Cypermethrin is a synthetic pyrethroid, which increases the permeability of sodium channels in invertebrate nerve membranes. This group has previously demonstrated that cypermethrin exposure at therapeutic treatment levels (5 μg/l) causes 50% mortality in shore crabs (*Carcinus maenas*) after 96 h. However, aqueous exposure to concentrations up to 500 ng/l and parenteral exposure to 10 ng cypermethrin did not cause any exposure related mortality. These data suggest that *Carcinus* is more resistant to cypermethrin than other non-target crustacean species tested. Sub-lethal aqueous and parenteral exposure was shown to induce glutathione *S*-transferase (GST) activity in *Carcinus*. hepatopancreas, suggesting a possible role for this enzyme system in cypermethrin metabolism.

Emamectin benzoate exerts its toxicity by increasing invertebrate membrane permeability to chloride ions. *Carcinus* and velvet crabs (*Necora puber*) fed Slice® coated salmon pellets to satiation suffered higher levels of mortality (18–32%, 96 h) than controls fed untreated pellets. In contrast to cypermethrin, GST activity in gill and hepatopancreas tissue of both species was not induced by oral exposure to emamectin benzoate after 4 or 7 days.

These data are discussed in relation to toxicant metabolism and the potential for developing tools to monitor for the biological effects of these chemotherapeutants in non-target biota.

A2.21 Mechanisms of mercury uptake by isolated rainbow trout intestine

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Although the transfer of mercury through aquatic food chains is well known, there is little information on the molecular mechanism of mercury uptake across the gut of fish. The aim of the experiments was to determine where mercury was absorbed in the gut and to characterise dose-dependent mercury uptake across the perfused intestine of rainbow trout (*Oncorhynchus mykiss*). The Na and Ca-dependence of mercury uptake was also investigated. Exposure of whole gut sacs to 100 μmol/l Hg as $HgCl_2$ in the luminal solution for 4 h caused Hg accumulation primarily in the mucosa (78% or more), with the intact mid and hind gut supporting 59% of the accumulated Hg. Total [Hg] of intact Hg-exposed tissues were (μmol/g dw, mean ± SE, n = 6):- oesophagus, 39.8 ± 9.7; stomach, 24.2 ± 8.4; pyloric caecae, 7.5 ± 2.1; mid intestine19.3 ± 5.2; and hind intestine, 26.6 ± 3.8 after 4 h exposure to 100 μmol l^{-1} luminal Hg, compared to between 0.1–0.7 μmol/g in control tissues (all tissues statistically different from controls, ANOVA, $P < 0.05$). Luminal exposure to [Hg] of 0 (control), 1, 10, 50, or 100 μmol/l for 4 h in perfused trout intestines showed saturable uptake rates (mean ± S.E., n = 3/dose) of 25 ± 6, 20 ± 6, 44 ± 18, 45 ± 14 and 44 ± 6 nmol/g dw/h respectively. This suggests carrier mediated transport into the gut cells and the blood. Additions of 2 mmol/l amiloride depressed Hg accumulation by the mid and hind gut by 40–50%, whilst additions of the Ca chelator 1 mmol/l EGTA increased Hg levels in the tissue.

A2.22 Abstract not supplied

A2.23 Reactive oxygen species and cell volume regulation in mammalian cells

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NIH3T3 mouse fibroblasts restore their cell volume following hypotonic cell swelling by release of ions (K^+ and Cl^-), organic osmolytes (taurine and other non-essential amino acids) and osmotically obliged water. Taurine, the zwitterionic, inert amino ethane sulphonic

acid, is accumulated by the active transporter TauT to a high degree in many cell types and taurine often constitute the major fraction of the cellular pool of organic osmolytes which are released upon hypotonic exposure. It has been demonstrated that a Ca^{2+}-insensitive phospholipids A_2 ($iPLA_2$) and the 5-lipoxygenase (5-LO) play a permissive role in the activation of the volume-sensitive taurine release pathway in NIH3T3 cells. Reactive oxygen species (ROS) are generated upon hypotonic exposure most probably by a NADPH oxidase and at a step down-stream to the $iPLA_2$ activation. Exposure to exogenous hydrogen peroxide increases the protein tyrosine phosphorylation of c-Src (Tyr^{527}) under hypotonic conditions and concomitantly potentiates the swelling-induced taurine release. The c-Src inhibitor PP2 also potentiates the swelling-induced taurine release. ROS have no effect on taurine release under isotonic conditions. It has been suggested that ROS modulate the swelling-induced taurine release once it has been activated by osmotic cell swelling and that ROS via oxidation and inactivation of protein phosphatases (PTP1B) reduce the activity of c-Src and consequently modulate the open probability of the volume-sensitive taurine release pathway (Lambert, Neurochemical Research, 29: 27–63, 2004).

A2.24 Effects of heavy metals on the haemocytes of the housefly

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The effects of several heavy metals; copper (Cu), zinc (Zn), cadmium (Cd) or lead (Pb) on the haemocytes of the housefly's (*Musca domestica* L.) blood (haemolymph) were studied under laboratory conditions. Larvae were exposed to low concentrations, similar to those detected in the soil of polluted areas in Poland, or high, semi-lethal concentrations. The total number of haemocytes, their types and morphological changes, and the expression of heat shock proteins HSP70 and HSP72 were studied in third-instar larvae reared on control media or media containing Cu^{2+} (5; 10,00 ppm), Zn^{2+} (100; 2,000 ppm), Cd^{2+} (3; 50 ppm) or Pb^{2+} (20; 10,000 ppm). We also studied heavy metal accumulation in adult flies from the same exposed populations of larvae.

Heavy metal concentrations in the body of flies showed a significant increase which depended on the concentrations in the rearing media. Most accumulation was detected after treatment with high concentrations of Cd and Pb, which increased the body content 30–40 times more than in tissues of control flies.

There were statistically fewer haemocytes, especially in larvae exposed to both low and high concentrations of Pb, as well as differences in their size and morphology. In addition, heavy metal-exposed insects had more prohaemocytes while the number of granulocytes was decreased. In the haemolymph there were numerous bacteria, reflecting the decreased phagocytic activity of the haemocytes. We did not detect, however, any clear changes in the expression of HSP70 and HSP72 in the cells, neither with low nor with high concentrations of any heavy metal studied.

A2.25 Hyperbaric oxygen therapy does not cause oxidative stress in isolated platelets: no effect on superoxide dismutase, catalase, or cellular ATP

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Hyperbaric oxygen (HBO) therapy is a recognised treatment for problem wounds, but it has been suggested that the therapy may cause oxidative stress in blood (Narkowicz et al. 1993, Free Rad. Res. Comm. 19, 71–80). Platelets were isolated from whole horse blood, and exposed to (A) 100% oxygen at 2.2 atmospheres, (B) 100% oxygen under normobaric conditions, or (C) air under normobaric conditions for 90 minutes. Samples were analysed before, then 0 h (immediately), 3 h, and 24 h post-treatment. There was no treatment-dependent effect on cellular ATP (Kruskal–Wallis, $P>0.05$; $n=6$), although all treatments showed a transient rise in ATP at 3 h post-treatment (e.g. in control platelets, median ATP 11.1 nmol/mg protein; range, 8.60–16.4; $n=6$; Kruskal–Wallis; $P<0.05$, compared to background levels of 8.75 nmol/mg protein). There was an expected cumulative increase in lactate content of the medium after 3h, but no differences between treatments over time (Kruskal–Wallis; $P<0.05$; $n=6$). There was no difference in platelet superoxide dismutase (SOD) activity between treatments (Kruska–Wallis, $P>0.05$), although all treatments showed a rise in SOD after 24 h in culture (e.g. control SOD at 24 h; median 0.24 units/mg protein; range 0.2–0.30; $n=6$) compared to initial background levels of about 0.18 units/mg protein. Catalase activity in platelets did not change over time, nor between treatments, and remained about 12 units/mg protein. This preliminary data suggests no detrimental effect on platelet biochemistry. Analysis of reduced glutathione levels, and lactate dehydrogenase release from platelets are in progress.