

Special Feature

CANADIAN NETWORK OF TOXICOLOGY CENTRES (CNTC) EXECUTIVE SUMMARY: ANNUAL RESEARCH SYMPOSIUM 20-21 MARCH 2001

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The Canadian Network of Toxicology Centres (CNTC) is a national, not-for-profit, university-based research program linking collaborating academic and government scientists in multi disciplinary research programs. The CNTC was established in 1992 under the auspices of an initial 5-year grant from Environment Canada. The CNTC gratefully acknowledges the outstanding support of Environment Canada, without whose continuing commitment (now in its 10th year), the CNTC national research initiatives would not be a reality. In addition to Environment Canada, current Network supporters include both the public and private sector. We also gratefully acknowledge the support of the Canadian Chemical Producers' Association, the Mining Association of Canada, Ontario Power Generation Inc., International Lead and Zinc Organization, and Nickel Producers' Environmental Research Organization.

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The CNTC infrastructure has included the following three research themes for the past four years: the Endocrine and Reproductive Ecotoxicology Program, the Risk Assessment Methodologies Program, and the Metals Speciation Program. The CNTC's administrative headquarters is on the campus of the University of Guelph in Guelph, Ontario, Canada. Organizationally, the CNTC functions through three regional research "nodes": the Toxicology Centre located on the campus of the University of Saskatchewan, the Centre for Toxicology located on the campus of the University of Guelph, and the Centre Interuniversitaire en Toxicologie, representing the interests of the Université du Québec à Montréal and the Université de Montréal.

The CNTC is incorporated as a charitable trust and is managed by a Board of Directors whose membership is drawn from the Canadian academic, public, and corporate sectors. In addition, the scientific excellence and relevance of the CNTC research programs are assessed through an annual review process by an external Expert Advisory Committee (EAC) who carry out a peer review of past progress and research proposals from all CNTC-sponsored scientists. External expert peer review is the sole responsibility of the distinguished panel of scientists from across North America with recognized expertise in areas directly related to the CNTC principal research themes. In fiscal 2000/2001, membership on the EAC included Dr. S. Kacew, University of Ottawa; Dr. R. DiGiulio, Duke University; Dr. J. Rodgers, Jr., Clemson University; Dr. J. A. Thomas (EAC Chair), University of Texas; Dr. P. Ross, Colorado School of Mines; and Dr. F. Wrona, National Hydrology Research Centre, Saskatoon. The EAC advises the CNTC Board of Directors of the outcome of its deliberations on review of research submissions. The CNTC's corporate public education and communications initiatives include: maintenance of a web site, publication of an annual newsletter, an annual report, and development of educational resources such as PERIL, a CD-ROM game targeted at high-school-level students and intended to demystify the concepts of risk, and an educator's guide on toxicology.

OVERVIEW OF RESEARCH PROGRAMS

Presentation of CNTC research programs at our 2001 annual research symposium included platform and poster presentations on 21 research projects, and participation from over 70 Canadian scientists, government and industry collaborators.

ENDOCRINE AND REPRODUCTIVE ECOTOXICOLOGY PROGRAM

Executive Summary

Understanding the risks posed by chemicals that have the potential to interfere with the normal functioning of the endocrine system and thereby contributing to alterations in growth, reproduction, and survival of humans and wildlife continues to be a priority on an international scale. Within Canada, the importance of this issue was evident at the 2000 Workshop on Establishing a National Agenda on the Scientific Assessment of Endocrine Disrupting Substances. Participants at the workshop identified the need for (1) a better knowledge of exposure, dispersal and effects of endocrine disrupting chemicals (EDCs) in the environment and (2) an improved understanding of how biochemical and physiological events resulting from exposure to EDCs relate to whole organism performance in terms of growth, development and

reproduction. Research supported by the CNTC and the Canadian Chemical Producers' Association has supported research that is contributing to our understanding of the risks posed by EDCs. Progress has been made in the three major research priorities established for the endocrine and reproductive ecotoxicology theme. These include:

- (A) Field assessment of reproductive, endocrine and immune modulation in wildlife.
- (B) Development and validation of screening tools for endocrine modulating substances.
- (C) Identifying mechanisms of modulation of reproductive function.

A. Field Assessment:

Studies conducted in fiscal year 2000-2001 have contributed to our understanding of the extent of endocrine disruption in feral populations of fish in Canada. This has included the development of new methods which can be used to identify potential EDCs in environmental samples and complex mixtures and in defining the mechanisms by which chemicals affect endocrine function and reproduction. These studies have surveyed a broad cross-section of the Canadian environment including waterways receiving effluents from sewage treatment facilities, agricultural practises and pulp mills, lakes contaminated with heavy metals, and broad geographical regions (St. Lawrence River, Great Lakes basin).

Studies conducted by Dr. D. Cyr and his collaborators have demonstrated estrogenic contamination of the St. Lawrence River which originates from different sources including municipal effluent. This was shown by the induction of estrogen-dependent proteins (vitellogenin) in both fish and molluscs. In related studies, Van Der Kraak and colleagues found that a range of fractions generated by preparative HPLC fractionation of a major Toronto sewage effluent contained material that bound to both estrogen receptors and androgen receptors. Collectively these studies have shown that sewage effluents may be a significant source of EDCs in the Canadian environment. Studies supported by the CNTC have shown that pulp and paper mill effluents contribute to a suite of adverse reproductive responses in fish including alterations in plasma sex steroid levels, reductions in gonadal size and delayed sexual maturity. Studies conducted over the past year have shown that endocrine active compounds as measured by their binding to the fish estrogen receptor, sex steroid binding protein or androgen receptor are rapidly accumulated by fish following exposure to bleached kraft mill effluent from two separate Canadian mills (Van Der Kraak - University of Guelph). These compounds were rapidly depurated from previously exposed fish that were transferred to clean water. Most investigators have assumed that persistent bioaccumulative compounds are responsible for the effects of pulp and paper effluent on endocrine function. These studies suggest that chemicals with very different properties may be responsible for the reproductive effects. Related studies have shown that fish exposed to pulp and paper effluent exhibit increased oxidative damage to membrane lipids raising the possibility that free radical generation may be a contributing factor to reproductive dysfunction.

Other studies conducted by Dr. A. Hontela (Université du Québec à Montréal) and her colleagues were among the first to show that long-term exposure to metals contribute to alterations in the functioning of the interrenal gland. This was associated with changes in the

secretion of cortisol and altered growth performance in fish. In studies conducted over the past year with yellow perch exposed to metals in the Abitibi region, there were changes in glycogen and triglyceride reserves and key enzymes of lipid and glucose metabolism. As such these studies are making an important link between endocrine changes, biochemical and whole organism markers of fitness.

(B) Development and validation of screening tools:

There is intense international demand for methods that can be used to assess the potential of chemicals to affect the endocrine system. This is an active area of research within the CNTC and studies conducted in fiscal year 2000-2001 have included the development and validation of methods to evaluate the effects of EDCs through both *in vitro* and whole animal *in vivo* approaches. This work is pivotal in defining the risks posed by individual chemicals and complex mixtures of environmental chemicals. Defining the utility of short-term markers of biological response (biomarkers) in relation to long-term developmental outcomes are fundamental to studies of EDCs. The studies conducted last year have provided broad biological coverage and included work with fish, amphibian, reptiles, birds, and mammals including human sex steroid receptors in culture.

Dr. P. Walfish (Mt. Sinai Hospital) is investigating the utility of yeast-based bioassay systems containing estrogen, androgen, thyroid hormone or retinoic acid receptor-reporter gene constructs to estimate endocrine activity. These studies have employed the latest molecular biology tools to construct the reporter genes, and the novel use of newly described transcription factors in an effort to refine these assays protocols such that they have the broadest utility in recognizing chemicals which interact with different nuclear receptor subtypes.

Dr. J. Chedrese (University of Saskatchewan) has investigated the use of an immortalized ovarian granulosa cell line to estimate the potential of chemicals to adversely affect ovarian function in mammals. The complex nature of the responses measured in these cells including steroidogenesis and proliferation (DNA synthesis) afford the possibility that these cells will have utility in identifying chemicals that act as ovarian toxicants via multiple mechanisms of action. As such, this assay may represent a significant improvement beyond receptor binding assays which are being used to screen chemicals and environmental mixtures for endocrine modulating activity.

Dr. P. Hodson (Queen's University) has used polyaromatic hydrocarbons (PAHs) as model compounds to investigate impacts on liver function, embryo larval toxicity and reproductive development in fish. Apart from providing new approaches to examining the cumulative risk of chemical exposure, this research is having immediate application in development of sediment toxicity bioassays.

Dr. J. Smits (University of Saskatchewan) and colleagues have been evaluating reproductive, endocrinological, and immunotoxicological methods of assessing the effects of PCBs in both first and second generational studies using the American kestrel. This work was instrumental in showing that PCB exposure is associated with delay in egg laying, smaller

clutches, decreased fertility, low nestling survival and altered behaviour along with a range of biochemical changes related to immune function and thyroid hormone synthesis.

Dr. V. Trudeau (University of Ottawa) has examined the effects of 4-octylphenol on hypothalamic gene expression in leopard frog tadpoles and the snapping turtle. These studies suggest that early changes in gene expression may be linked to disrupted hypothalamic function in later life.

Dr. G. Van Der Kraak (University of Guelph) has tested the effects of indomethacin which is an over the counter pharmaceutical used to inhibit cyclooxygenase, on reproductive endocrine function in the goldfish. Indomethacin was shown to reduce plasma testosterone levels when administered via the water at levels recently reported in the Canadian environment. This shows that pharmaceuticals (other than birth control pills) affect the general physiology and endocrinology of non-target species.

(C) Identifying mechanisms of modulation of reproductive function:

All of the studies listed in Section B address the issue of mechanisms of action but studies conducted in 2000/2001 also included studies that are not screening protocols *per se* but rather research that addresses specific hypotheses that identify mechanism or cellular targets that may be responsible to environmental chemicals. Importantly, these studies have moved beyond examining the effects of estrogens or androgens and have considered other mechanisms that could contribute to alterations in fitness. This includes immune system interactions, the role of oxidative stressors, and changes in the expression of specific gene products that may mediate the response to environmental chemicals and lead to developmental and reproductive effects.

Dr. N. Bols (University of Waterloo) has been evaluating the hypothesis that environmental chemicals affect reproduction by influencing the activity of macrophages that in turn, through the production of reactive oxygen species and cytokines, influence gonadal physiology. Studies to date have shown that reactive oxygen species and nitric oxide do not mediate the effects of dioxin on macrophage activity.

Dr. P. Krone (University of Saskatchewan) has been using zebrafish embryos as a model system to evaluate the potential of environmental chemicals to affect developmentally regulated genes and cell populations. The cloning of the Vasa gene which is a marker of primordial germ cells and is providing an opportunity to examine the effects of EDCs on gonadal development.

Studies by Dr. A. Hontela and her colleagues have shown that the interrenal gland is a target for pesticides and metals which have adverse effects on corticosteroid secretion. This work has been instrumental in identifying the interrenal gland of fish as a target for EDCs.

Highly Trained Personnel:

One of the important features of the CNTC program that deserves special mention is that of the training of high quality personnel. The projects in the Reproductive and Endocrine

Ecotoxicology Program provided support for 32 students at the undergraduate and graduate levels and 12 postdoctoral fellows in fiscal year 2000-2001. Most of the projects are interdisciplinary in nature and afford a broad learning experience for the students.

Endocrine and Reproductive Ecotoxicology Program Platform Presentations

Macrophages as targets of environmental contaminants and mediators of reproductive dysfunction

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One mechanism by which environmental contaminants cause reproductive dysfunction is through their modulation of macrophages. The environmental contaminants examined were the polycyclic aromatic hydrocarbons (PAHs) and the dioxin-like compounds. Some of these contaminants, such as phenanthrenequinone (PHQ), can stimulate the production of reactive oxygen species (ROS) by cells in general and by macrophages in particular, leading to oxidative stress. Treating cell suspensions from the rat corpus luteum with PHQ led to a pronounced increase in ROS production, enhanced further by concurrent treatment with luteinizing hormone (LH). Progesterone secretion by corpus luteal cells was slightly stimulated by PHQ. By contrast, PHQ caused a dramatic inhibition in progesterone secretion by LH-treated corpus luteal cells. The parent PAH, phenanthrene, caused no change in ROS formation or progesterone secretion under any conditions. Therefore, the effect on progesterone secretion by PHQ appeared to be mediated by ROS. PHQ also caused changes in ROS formation by macrophages. A rainbow trout macrophage cell line, RTS11, responded to dioxin-like compounds with little or no induction of 7-ethoxyresorufin o-deethylase activity, whereas strong induction was observed with the rainbow trout liver cell line, RTL-W1. However, coculturing RTS11 with RTL-W1 reduced EROD induction by RTL-W1. These results suggested that macrophages modulate the response of other cells to dioxin-like compounds. In reproductive ecotoxicology, the effect of contaminants on the interaction between macrophages and steroidogenic cells will be of special interest.

Molecular mechanisms mediating the effects of endocrine disrupters in the ovary

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We investigated the effects of DDE, the major and most stable metabolite of DDT, and the DDT analog, methoxychlor (MXC), on ovarian function. These endocrine disrupters (EDCs) are known for their reproductive toxicity. We tested the hypothesis that DDE and MXC mimic the natural estrogen estradiol-17 β in the ovary. We also investigated two drugs currently used in the formulation of oral contraceptives, the progestin levonorgestrel (LNG) and the synthetic estrogen ethinyl-estradiol (E-E2), found contaminating the environment through household

sewage. Another EDC, the gamma isomer of the hexachlorocyclohexane ((HCH) which was recently reported to inhibit testicular steroidogenesis, was also studied.

We observed that DDE mimics only some of the effects of estradiol-17 β , such as stimulation of granulosa cell proliferation. Inhibition of progesterone synthesis appears to be unrelated to the estrogenic effect of DDE. DDE, at 300 nM and higher concentrations, inhibits the protein kinase-A-stimulated cAMP generation in granulosa cells. Estradiol-17 β , on the other hand, does not affect protein kinase-A-stimulated cAMP, induced by either FSH or cholera toxin, at any of the concentrations tested in our study. Similar results were observed when the effect of DDE and estradiol-17 β were tested on FSH- and cholera toxin-stimulated cAMP in CHO cells expressing the FSH-R. MXC, which is considered a proestrogen, does not affect granulosa cell proliferation, but inhibits progesterone synthesis. LNG and E-E₂ stimulates progesterone synthesis, an effect not altered by DDE at concentrations up to 300 nM. However, MXC (1 to 10 :M) and (HCH (30 :M) inhibited LNG- and E-E₂-stimulated progesterone synthesis.

Overall, we concluded that combinations of EDCs may interact and affect reproductive endocrinology. EDCs can also affect the therapies associated with the use of FSH in the course of ovarian stimulation and the effects of oral contraceptives.

The presence of xenoestrogens in the St. Lawrence in proximity of the Island of Montreal: where are they coming from and should we be concerned?

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The presence of environmental estrogens in the aquatic ecosystem has been reported to represent a significant threat for aquatic species such as fish. In addition, contamination of aquatic ecosystems by estrogenic substances could result in effects on birds and mammals that eat fish, including humans. The objective of this study was to determine the presence of estrogen mimics in proximity to the Island of Montreal and to determine if Montreal Urban Community sewage effluents contribute to the pollution of the St. Lawrence by releasing estrogenic substances into the river. Spottail shiners (*notropis hudsonius*) were captured at five sites on the St. Lawrence in proximity to the Island of Montreal and used to determine if these fish were exposed to estrogenic substances. Coliform counts indicated that Ilôt Vert and Contrevoeur were exposed to municipal effluent and that levels at Ilôt Vert were the highest. Total cellular RNA was isolated from whole fish livers and subjected to semi-quantitative RT-PCR. Results indicated that VTG mRNA levels were lowest at Ile de la Paix, which received water input primarily from the Great Lakes. VTG mRNA levels were significantly higher at all the other sites indicating that these fish were being exposed to estrogenic substances. Non-specific immune function was decreased at all sites upstream from the MUC effluent where VTG and mRNA levels were increased. Short-term exposure of immature rainbow trout to MUC sewage effluent did not result in induced VTG mRNA levels. Together, these data suggest that there is an important contamination, which encompasses almost 70 kms, of the St. Lawrence River by estrogenic substances. Furthermore, our results suggest that this contamination may originate in

the Ottawa River. The toxicological consequences of this contamination on fish and mammals that eat contaminated fish are currently being determined.

Toxicity of alkyl-substituted PAH

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Our objective was to synthesize alkylphenanthrene compounds, test their toxicity to early life stages (ELS) of fish, develop analytical techniques to measure their concentrations in water and tissue, and formulate structure toxicity relationships. Alkyl-PAH occur in crude oil, coal tar, and creosote and are sufficiently persistent that they are enriched as PAH mixtures weather. The toxicity of Exxon Valdez crude oil to ELS of pink salmon and Pacific herring has been correlated with concentrations of C1-C4 phenanthrenes in interstitial water. We found that one compound, retene (7-isopropyl-1-methylphenanthrene), caused similar symptoms as the mix of C1-C4 phenanthrenes, including induction of CYP1A enzymes. While retene was accumulated and excreted rapidly (1/2-life of 14 h), inhibiting CYP1A monooxygenase activity increased tissue retention but reduced toxicity. Conversely, accelerating the metabolism of phenanthrene by inducing CYP1A enzymes with BNF increased excretion rates, reduced tissue concentrations, but increased toxicity. Therefore, the toxicity to fish of alkylphenanthrenes should vary with the location and size of alkyl substituents, as they affect Kow, bioaccumulation, the nature and number of metabolites, and the extent of CYP1A induction.

To model and predict the risk of BSD and recruitment failure due to alkylphenanthrenes, we initiated research to develop structure activity models. In tests with early life stages of medaka, 1-methylnaphthalene and 2-pentyl-phenanthrene were non-toxic at 10-1,000:µg/L; for other compounds, potency for affecting hatch or BSD increased in the order of phenanthrene < 1-methylphenanthrene < 2-ethylphenanthrene < retene (1,7-dimethyl and 7-ethyl-1-methyl were not yet tested). The affinity of alkyl- and un-substituted PAH for binding to trout arylhydrocarbon receptor (AhR) was assessed by measuring displacement of radiolabelled 2,3,7,8-TCDD from two rtAHR isoforms. The rank order of receptor binding was the same as the rank order of potency for CYP1A induction in vivo and the rank order of chronic toxicity to larval trout and medaka. Hydroxylation of an alkyl side-chain suggested a mechanism that might explain differences in toxicity between un-substituted and alkyl-PAH. Recent toxicity tests suggested an endocrine effect; medaka exposed to retene during sexual maturation showed impaired spermatogenesis, testis-ova, and fin erosion, similar to effects caused by MESA crude oil.

Mechanisms of action of pesticides and metals on adrenal steroidogenesis and intermediary metabolism in teleost fish.

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Our CNTC funded research provided important new data to elucidate the mechanisms of adrenotoxicity in teleost fish and compare the activities of several environmentally relevant toxicants.

We found evidence that Cd disrupts the adrenal steroidogenic pathways upstream from the step where pregnenolone is used in the enzymatic cascade leading from cholesterol. The role of calcium in Cd-induced adrenal toxicity was also investigated in our teleost model using nifedipine (a blocker of calcium channels) and BAY K 8644 (calcium channel agonist). Preliminary experiments suggested that nifedipine increases the toxicity of Cd within the steroidogenic pathways. These experiments will identify the intracellular processes targeted by Cd within adrenal steroidogenic cells.

We completed an *in vivo* study with endosulfan, a chemical stressor, in rainbow trout. Fish were exposed for 30 days and their endocrine, metabolic and physiological responses were characterized. To initiate our studies on oxidative stress induced by pesticides, we measured in adrenal cell suspensions exposed to endosulfan *in vitro*, the activity of catalase. Dose-dependent effects were observed, suggesting that oxidative stress may be a mechanism through which endosulfan exerts its adrenotoxic effects. The methods for assays of other indicators of oxidative stress (SOD, GPx, glutathione, malondialdehyde) were validated for use in trout cells exposed to endosulfan.

We completed a large-scale field study (complementary to our work within MITE-RN) with yellow perch exposed to metals in the Abitibi region. Significant exposure-related differences in energy reserves and enzyme activities were observed. We detected an important seasonal cycle in the build up of liver glycogen and triglycerides, and in enzyme activity in fish from reference lakes, while such seasonal cycles were not observed in fish from polluted lakes. Our data suggested that chronic metal exposures may cause a metabolic impairment, in addition to hormonal anomalies. Differences in muscle LDH, related to metal burdens and diet were also observed (Sherwood et al., in prep). Since diet may also influence metabolism, the effects of metals on intermediary metabolism will be investigated under controlled laboratory exposures to metals.

Effects of putative endocrine disrupting substances on zebrafish development.

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It is now recognized that a variety of chemicals released into the aquatic environment are capable of disrupting endocrine function in fish and other vertebrates. While it is thought that some of the effects observed in exposed adults may arise by permanent organizational changes occurring during embryogenesis, little work has addressed the effects of these compounds in developing embryos. The emergence of zebrafish as a model developmental system has prompted us to utilize it to assess cell populations and genes affected by endocrine modulators, and in particular environmental estrogens. We have previously utilized antisense RNA probes to mRNA encoding the primordial germ cell (PGC) specific marker *vasa* in order to examine the early migration and distribution of PGCs in embryos exposed to endosulfan and nonylphenol via whole mount *in situ* hybridization analysis. The data revealed that these compounds cause alterations in the distribution of PGCs along the anterior-posterior axis in 24-hour-old embryos, suggesting that there are early embryonic cell types that are affected by these compounds. In order to further develop the zebrafish a model system for the rapid assessment of early embryological targets of putative EDSs, we have initiated an analysis of cell-specific, stress-inducible gene expression in transgenic zebrafish. This information is critical in order to allow for proper interpretation of toxin-inducible expression in experimental embryos during subsequent experiments. Importantly, we are able to assess gene expression in living embryos using the green fluorescent protein (GFP) assay. Thus, we can directly correlate stress-induced gene expression in specific cell types with the subsequent impact on development in the same embryo.

Exposure to xenoestrogens and UV radiation disrupt hypothalamic function and metamorphosis in the leopard frog tadpole.

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Worldwide declines in amphibian populations, including the Leopard frog in Canada, likely have several causes. Habitat loss is one contributor. Increased UV-B radiation because of ozone depletion and contamination of wetlands with pesticides and other pollutants induces developmental abnormalities, and thus, are suspected causes. Few investigators have examined the mechanisms behind the effects of UV-B or pollutants. Early exposure of the developing Leopard frog tadpole to combinations of UV-B and OP disrupt metamorphosis by altering growth and age at limb development. The control of all aspects of growth and metamorphosis is mediated by the hypothalamus in the brain. Neurotransmitters and neuropeptides produced there regulate pituitary and thyroid hormone release, the ultimate mediator of the metamorphic process. In frogs with neurotransmitter synthesis, angiogenesis and growth factor signaling that are disrupted by responses by determining changes in developmental sensitivity to UV-B and/or OP. The power of the tadpole model is that we can correlate disruption of hypothalamic gene expression patterns with altered growth and viability of the newly metamorphosed froglets.

Developing methods to assess reproductive endocrine function in fish.

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The workshop "Establishing a National Agenda on the Scientific Assessment of Endocrine Disrupting Substances" identified the needs for (1) a better knowledge of exposure, dispersal and effects of EDCs in the environment and (2) an improved understanding of how biochemical and physiological events resulting from exposure to EDCs relate to whole organism performance in terms of growth, development and reproduction. Our research sponsored by the CNTC and the CCPA over the past year responded to these needs by supporting the development, validation and application of *in vitro* assays through to whole animal protocols to evaluate the effects of chemical exposure on reproductive and endocrine physiology of fish. The new tools developed were radio-receptor assays to quantify binding to the retinoic acid receptor as well as methods which can be used to evaluate the effects of EDCs on to determine the effects of xenobiotics on gonad steroid biosynthesis and metabolism (aromatase assays and measurement of the steroid acute regulatory protein). Research over the past year contributed to the identification of EDCs in pulp mill, sewage effluents and agricultural runoff and to the characterization of the activity of alkylphenol polyethoxylate derivatives which are the subject of a PSL2 evaluation. Studies have provided insight into novel mechanisms (induction of oxidative stress) by which chemicals may affect fish reproduction. This work provides the tools to assess the extent of endocrine disruption in feral populations, identifying potential endocrine disrupting chemicals in environmental samples and complex mixtures, and understanding the mechanisms by which chemicals affect endocrine function and reproduction.

Hormone dependent nuclear receptors function as transcription factors that bind to upstream DNA enhancer elements of target genes to control growth, development and homeostasis. Preliminary studies in yeast-based assay systems demonstrated the feasibility of determining rank order gene activation potencies EC_{50} of natural hormones compared to synthetic or environmental compounds induced by specific hormone-dependent nuclear receptor isotopes. Further studies were in progress to determine the ultimate utility of *in vivo* yeast-based reporter gene assays and *in vitro* protein-protein analyses which measure the formation of hormone dependent dimeric and trimeric gene activation complexes in the detection of potential environmental endocrine disruptors that may either mimic hormone-induced effects (i.e. function as agonists) or interfere with hormonal action (i.e. function as antagonists).

Endocrine and Reproductive Ecotoxicology Program Poster Presentations

Development of a retinoic acid receptor-binding assay with rainbow trout tissue: the influence of environmental contaminants on retinoic acid binding and its receptors.

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We have developed a competitive binding assay with fish tissue and the radio-labelled all-trans and 9-cis isomers of retinoic acid (RA). Specific binding, indicative of receptor binding, was found in all rainbow trout tissues tested. In the gills and liver, all-trans RA bound with high affinity, while 9-cis RA bound with lower affinity and higher capacity. We have identified the two classes of RA receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The gill with primarily RARs, and the liver with primarily RXRs were used in a series of tests examining the effects of contaminants on RA binding. Methoprene acid, an insect growth regulator, competed with 9-cis RA for binding to the RXRs of the liver. Exposure of white suckers to pulp and paper mill effluent resulted in sex specific responses; in the liver, males had elevated RA binding levels, while in females it was depressed. The ovaries did not experience as great a decrease in RA binding levels as did the liver. The mechanisms underlying these changes in RA receptor levels are currently under investigation and may represent a new theme in the studies of endocrine disruption.

Effects of eating fish from an xenoestrogen-contaminated environment on the postnatal development of the male reproductive system

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It has been suggested that male mammalian reproductive health is declining and that environmental contaminants may be contributing to this decline. Studies from our laboratory indicate that the St. Lawrence River in proximity to the Island of Montreal is contaminated by xenoestrogens. We have reported that immature male spottail shiners have induced vitellogenin mRNA levels. The most important induction in VTG mRNA levels was found in shiners captured at Ile Beauregard where sediment levels of nonylphenols are elevated. The objective of this study was to determine whether or not mammals, including humans, which eat fish that come from an environment containing xenoestrogens could alter the development of the male reproductive system. Pregnant Sprague-Dawley rats were purchased two weeks prior to parturition. At the time of birth, male pups were randomly divided and placed with a lactating female. Mothers were administered saline or homogenized fish (1% body weight) three times every week during lactation. Three experimental groups were used: saline control, non-VTG positive shiners (Ile de la Paix) and VTG-positive shiners (Ile Beauregard). Pups were sampled either at 21 days (weaning) or as adults (day 91). There were no significant effects on body weights between treatment groups throughout the experimental period. At 21 days the weights of the testis and epididymis were not different between groups and there were no differences in size of the seminiferous tubules of the testis. In adult rats, there was a significant decrease in the

weight of the epididymis and while testicular weights were also decreased these were not significant. Sperm motility was assessed using a computer assisted semen analyzer. Results indicate that there was a significant decrease in the percent of motile sperm in rats given VTG-positive fish. There were no effects on other parameters of motility suggesting that motile sperm were normal. These data indicate that lactational exposure to contaminants from VTG-positive fish may also affect the reproductive function of the male when they reach adulthood.

Effects of *in utero* exposure to tributyltin on sperm motility in adult rats

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Tributyltin (TBT) is an environmental contaminant commonly used in anti-fouling agents for boats, as well as by-product from several industrial processes. TBT has been shown to be immunotoxic, however, little information exists regarding the effects of TBT on mammalian reproduction. The objective of this study was to determine the effects of TBT on sperm motility, in male offspring exposed *in utero* to TBT. Female Sprague-Dawley rats were impregnated and exposed to TBT (0, 2.5, 10, 20 mg/kg) by gavage throughout gestation. The pups were weaned on day 21 and sacrificed at day 91 for sperm analyses using an IVOS sperm analyzer. There were 6 animals per group with the exception of the high dose where only 2 animals survived treatment. Body weights of TBT treated rats were 10 and 30% lower than controls in the 10 and 20 mg/kg groups respectively. While testis weights were slightly decreased by TBT these differences were not significant. Sperm from the cauda epididymidis were removed and were assessed for a variety of parameters including motility, concentration, and progressive velocity. Sperm motility was decreased by 15% in the 10mg/kg group and 21% in the 20 mg/kg. Average track speed of the sperm was also increased in the treated groups. There were no significant differences in other parameters such as beat frequency and lateral amplitude. These results suggest that exposure to TBT *in utero* can result in decreased sperm function when the animal reaches adulthood. Supported by the Toxic Substances Research Initiative.

Effects of endosulfan, an organochlorine pesticide, on adrenocortical cells in rainbow trout (*Oncorhynchus mykiss*)

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The effects of endosulfan on the cortisol steroidogenic pathway and the antioxidant defence system were investigated in rainbow trout (*Oncorhynchus mykiss*). Enzymatically dispersed adrenocortical cells were exposed *in vitro* to endosulfan and cortisol secretion in response to ACTH (adrenocorticotrophic hormone) or dbcAMP (intracellular second messenger), as well as cell viability were determined. The effective dose, EC50 (dose that inhibits cortisol secretion by 50%), the median lethal dose, LC50 (dose that kills 50% of the cells) and the LC50/EC50 ratio were established to assess the endocrine toxicity of endosulfan. In comparison, other pesticides tested in our laboratory using the same methodology were ranked as follows : EC50, Endosulfan < Diazinon < Mancozeb < Atzazine ; LC50, Diazinon < Endosulfan < Mancozeb < Atrazine. To compare the effects of endosulfan and its metabolites on cortisol

secretion following *in vivo* exposition, fish were exposed orally (gelatin capsule, *per os*) to endosulfan for 30 days. Dose-dependant effects of endosulfan on cortisol secretion by isolated adrenal fragments from fish exposed *in vivo* were observed.

To investigate the mechanisms of action of endosulfan in teleost steroidogenic cells, Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Transferase (GST) activities, reduced (GSH) and oxidized (GSSG) glutathione levels, lipid peroxidation, cortisol secretion and viability will be measured after exposition *in vitro* to endosulfan. Protocols for measurements of enzyme activities in the steroidogenic cells of rainbow trout were developed in our laboratory. Preliminary results indicate a significant increase of CAT activity, 120% of non-exposed control, at 10^{-6} M endosulfan, a dose that inhibits cortisol secretion to 80% of the control. When the dose of endosulfan was increased to 10^{-4} M, CAT activity and cells viability were reduced and cortisol secretion was undetectable. These preliminary results suggest that a specific and saturable antioxidant defence system is activated at non-cytotoxic doses of endosulfan (study funded by CNTC).

Modulation of amphibian immune response by agripesticides at environmentally relevant concentrations

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The hypothesis to be tested in this project is to verify if the resistance of amphibians could be significantly impaired by agricultural contaminants, which could prevent the immune system from providing adequate defense. Among the chemical-induced immunotoxic effects that have been reported, immunomodulation remains at the top of the list. To study this aspect and to take into account the structural and functional complexity of the immune system, various immunological assays were adapted, validated and used to evaluate the immune status of *xenopus laevis* and *rana pipiens* following *in-vivo* exposure to a representative mixture of agripesticides in a dose-response fashion. Young adult *xenopus laevis* and *rana pipiens* were exposed through water (24/24 hours for *xenopus*; 14/24 hours for *rana*) for 21 days. The representative mixture was composed of aldicarb, atrazine, dieldrine, endosulfan, lindane and metribuzin. The concentrations selected were environmentally relevant. The mixture was prepared with dmsO and dechlorinated water. Following the exposure, viability of splenocytes, phagocytosis and mitogenic assay were performed. The viability was not significantly influenced. However, we have noted that the splenocytes from *rana* seem to be more fragile showing a maximum viability of about 80% compared to about 95% for *xenopus*. Absolute number of phagocytes were significantly modified for both species at concentrations ten times lower than those measured in the environment. Moreover, the mixture has severely suppressed the mitogenic response to conA and pha in *rana* for all the treated groups. following the exposure of both species of frogs to the mixture of agripesticides, we have shown that the mixture was not cytotoxic but impaired significantly the immune system of frogs by modulating the phagocytosis as well as the basic capability of lymphocyte to proliferate.

This work was supported by a grant from toxic substances research initiative.

Culture of fetal thymic organ as an organ assay for endocrine disruptors

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Fetal thymic organ culture (FTOC) has been developed to investigate early events in the thymus, such as colonization of the epithelial anlage by hematopoietic precursors and the interaction between primitive T cell precursors and the thymic microenvironment which ultimately leads to the development of a fully functional thymus and mature T cell. Because of the endocrine involvement in the process and because the particular sensitivity to chemical of this critical lifestage, it was postulated that FTOC may represent a good candidate as an organ screening assay for putative endocrine disruptors. With this goal in mind we decide to determine the endocrine effects on thymocyte differentiation, by first assessing gender differences. Then, *in vitro* exposure was carried out to a range of the two native hormones E_2 and T_3 , with subsequent analysis of T-cell differentiation using the CD4 and CD8 markers expression on cell surface. We also establish baseline data on the location and expression of cell adhesion molecules a catenin, b catenin, and E-cadherin to allow us to determine if endocrine induced changes in thymocyte differentiation involved changes in cell to cell communication. Baseline data on the expression of estrogen receptors (a and b) and thyroid receptors (a and b) was also necessary to establish the link between experimental treatments and alterations in thymocyte differentiation. Microdissection of the developing mouse embryo was performed to examine the gonads and determine the sex of the animals. With the criteria for gender discrimination established, no sex difference was found in CD4 or CD8 expression between male and female as well as in any treated or untreated thymic lobes with hormones. For the addition of hormones, estradiol caused in both sex a significant decrease in the proportion of $CD4^-CD8^-$ thymocytes and a slight concurrent increase in the $CD4^+CD8^+$ population. Triiodothyronine caused a decrease in the percentage of single positive CD4 thymocytes. The decrease in CD4 percentage also resulted in an decrease in the ratio of CD4 to CD8 thymocytes. Using immunocytochemistry, we can identify A and B catenin, and E-cadherin in the developing thymus and have begun to localize the expression of these factors. We have shown that the catenins are generally distributed between all cell types in the thymus with no bias for medullary or cortical areas. E-cadherin, on the other hand, appears to be restricted to the medullary region of the developing thymus. These results demonstrate the sensitivity of this organ assay to variation to endocrine moiety. Next step will be to challenge the cultures with a selection of endocrine disrupting chemicals.

This work was supported by a grant from Toxic Substances Research Initiative.

Developmental toxicology in transgenic zebrafish: Molecular assessment of sensitive cell populations in live embryos

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It is now recognized that a variety of chemicals released into the aquatic environment are capable of disrupting endocrine function in fish and other vertebrates. While it is thought that some of the effects observed in exposed adults may arise by permanent organizational changes occurring during embryogenesis, little work has addressed the effects of these compounds in developing embryos. The emergence of zebrafish as a model developmental system has prompted us to utilize it to assess cell populations and genes affected by putative endocrine disrupting substances.

Our laboratory has been examining the expression of heat shock protein (hsp) genes during early zebrafish development. We have previously cloned and characterized members of the *hsp10*, *hsp47*, *hsp60*, *hsp70*, and *hsp90* gene families, and shown that they are expressed in unique cell and developmental stage specific expression patterns. In previous work by others, *hsp70* expression has been examined as a potential biomarker for toxin exposure in adult tissues and cell lines. We have found that two putative EDSs known to have an impact on early germ cell development (endosulfan and nonylphenol) also stimulate the expression of the *hsp70* gene in cultured embryonic zebrafish cell lines. In the present study, we report the development of transgenic zebrafish as a model system for the rapid assessment of early embryological targets of putative EDSs. These fish carry the promoter for the gene encoding heat shock protein 70 (*hsp70*: the stress-inducible member of this gene family) linked to the report gene encoding green fluorescent protein (GFP). Two strains of these fish have now been established in our laboratory for use in toxicological analysis. We are currently examining the normal patterns of *hsp70* gene expression and how they compare to the expression of the transgenes. This information is critical in order to allow for proper interpretation of toxin-inducible expression in experimental embryos during subsequent experiments. For example, we have found that the endogenous *hsp70* gene is expressed for a short window of time during normal lens development, a pattern that recapitulated in one of the transgenic strains. Importantly, we are able to assess gene expression in living embryos using the GFP assay. Thus, we can directly correlate stress-induced gene expression in specific cell types with the subsequent impact on development in the same embryo. (Supported by NSERC and CNTC).

Mechanisms of action of cadmium in adrenal steroidogenesis of teleost fish: Evidence for involvement of calcium channels.

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Calcium entry into the steroidogenic cells involves voltage-dependent calcium channels. Since calcium is required for a normal cortisol secretion and heavy metals such as cadmium (Cd) are susceptible to interfere with this important second messenger, we investigated the interactions of Cd with calcium. The mechanism of action of Cd in the adrenal steroidogenesis in a teleost

fish, Rainbow trout (*Oncorhynchus mykiss*), was investigated using nicardipine, a calcium channel blocker and BAY K8644, a calcium channel agonist. Cd inhibited ACTH-stimulated cortisol secretion in a dose-dependent manner (EC50, concentration resulting in 50% inhibition = 1×10^{-4} M). At this concentration, pregnenolone-stimulated cells maintained the cortisol secretion at the control level, indicating that Cd disrupts the signalling pathway at steps prior to the conversion of cholesterol into pregnenolone. Nicardipine inhibited ACTH- and pregnenolone-stimulated cortisol secretion in a concentration-dependent manner, demonstrating that voltage-dependent calcium channels are involved in stimulated cortisol secretion. Pretreatment of cells with nicardipine at IC50 (25 mM) and exposure to cadmium (1×10^{-5} to 1×10^{-2} M) increased the inhibition of ACTH-stimulated secretion by Cd, suggesting that blocking calcium entry increased Cd toxicity. Although BAY K8644 did not have an effect on cortisol secretion, pretreatment of cells with this agonist and exposure to Cd increased the inhibition of ACTH-stimulated secretion but not pregnenolone-stimulated secretion. These results suggest that BAY K8644 may allow Cd entry at the same time as calcium, thus increasing toxicity of Cd. The effects of heavy metals have never been investigated in the signalling pathways in fish adrenal steroidogenic cells. Our study on calcium/cadmium influx into steroidogenic cells will allow us to discover the mechanism by which Cd interferes with the homeostasis of intracellular calcium.
(Study funded by CNTC)

Effects of Atrazine on Type III deiodinase mRNA levels in premetamorphic *Xenopus Laevis*

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It has been suggested that the global decline of amphibian populations results from exposure to agrochemicals present in the environment. Previous studies have reported that atrazine, a widely used herbicide, has adverse effect on thyroid function in amphibians. The objective of this study was to determine whether or not atrazine could alter deiodinase mRNA levels in premetamorphic *Xenopus laevis*. Deiodinase type III (5D) was cloned from the tail of premetamorphic *Xenopus* using an RT-PCR approach. A 428 bp sequence containing a portion of the coding region of the 5D was isolated and its identity was confirmed by sequencing. This enzyme is responsible for converting thyroxine to its inactive metabolite reverse triiodothyronine. Using GADPH as a control, a semi-quantitative RT-PCR approach was established for 5D. Premetamorphic *Xenopus* tadpoles (stage 55) were exposed to 21 $\mu\text{g/L}$ of atrazine for 48 hrs at 21C. At the end of the exposure period, the animals were terminally sampled and the tail was isolated and frozen in liquid nitrogen. Total RNA was isolated from the tail, treated with DNase and used to quantify 5D mRNA levels. Results indicate that exposure to atrazine did not significantly alter 5D. To further assess whether tail resorption and cellular apoptosis was induced in the *Xenopus* tail, DNA was isolated from this tissue and DNA fragmentation measured. Results indicate that atrazine did not induce apoptosis in the *Xenopus* tail. These results indicate that atrazine does alter 5D mRNA or stimulate apoptosis in premetamorphic *Xenopus* tadpoles. Supported by TSRI.

Seasonal variation in carbohydrate and lipid metabolism of cortisol-impaired METAL-EXPOSED YELLOW perch (*Perca Flavescens*)

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Previous studies by our research team reported that yellow perch chronically exposed to metals in the mining region of Abitibi have a reduced growth efficiency (expressed as consumed food mass in relation to growth) and delayed gonadal maturation. The present study was designed to test the hypothesis that the effects of metals on growth may be caused by a hormonally-mediated metabolic imbalance.

The effects of heavy metals on intermediary metabolism and enzyme activity were investigated in yellow perch (*Perca flavescens*), sampled from lakes situated along a contamination gradient of Cd, Zn and Cu in the mining region of Rouyn-Noranda, Québec. Fish were captured in fall and summer, and they were sampled before or after a confinement of 1 hour. An exposure-dependent decrease in condition and an impairment of the capacity to increase blood cortisol and glucose following the confinement were observed. In the summer, fish from the most contaminated lakes had lower hepatic triglycerides and glycogen reserves, and lower activities of malic enzyme (ME) and glucose 6-phosphate dehydrogenase (G6PDH), two key enzymes involved in lipid metabolism, than did fish from reference lakes. There were no differences in plasma free fatty acids. In the fall, fish from contaminated lakes exhibited perturbed glycogen reserves and had lower plasma free fatty acids levels, lower triglycerides reserves, and higher activities of pyruvate kinase (PyK), a glycolytic enzyme, glutamate oxaloacetate (GOT), phosphoenolpyruvate carboxykinase (PEPCK) and malate dehydrogenase (MDH), gluconeogenic enzymes, than fish from reference lakes. The results of this study indicate that a chronic exposure of yellow perch to sublethal levels of heavy metals impairs growth and alters the seasonal cycling of liver glycogen and triglycerides as well as the capacity to utilize these reserves. (Study funded by CNTC, metal analyses funded by MITE-RN)

A possible mechanism of pulp mill effluent induced reproductive dysfunction in White Stucker (*Catostomus commersoni*)

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Numerous studies over the past decade have identified impaired reproduction in feral white sucker and other fish species exposed to pulp mill effluents. These effects are consistent despite altered bleaching techniques and effluent treatment strategies suggesting that a common constituent or mechanism contributes to the toxicity of the various mill effluents. Free radicals can potentially be produced by a number of effluent constituents and while their impact on fish has not been thoroughly investigated, mammalian lab studies have demonstrated a range of reproductive and somatic dysfunctions with exposure. We have conducted studies to evaluate the involvement of free radicals in mill effluent toxicity. Feral white sucker exposed to 50%

bleached mill effluent (BME) for 4 days showed a modest increase in hepatic free radical damage as quantified by 2-thiobarbituric acid reactive substances (TBARS). The same effluent in 21 day lab exposures using immature rainbow trout (*oncorhynchus mykiss*) resulted in a dose-dependent 30-90% increase in hepatic TBARS which was strongly correlated to an induction of mixed function oxidase (MFO) activity. While MFO detoxification enzymes are known to produce free radicals, they are largely hepatic in distribution and cannot explain extra-hepatic lipid peroxidation as previously observed in gonadal tissue. In addition, we have shown that redox-active compounds could also contribute to lipid peroxidation as i.p. injections of ferric nitrilotriacetate (15 mg/kg body weight) resulted in significant increases in hepatic free radical damage; a 2 hour incubation resulted in levels similar to that observed in effluent exposed fish. These findings suggest elevated free radical activity could be a common mechanism of toxicity for fish exposed to pulp mill effluent.

The environmentally persistent Xenoestrogen Octylphenol (OP) upregulates the expression of members of the Amyloid Precursor Protein Family in the hypothalamus of the common Snapping Turtle, *Chelydra serpentina serpentina*.

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The gonadal estrogen estradio-17b (E2) is important for the development and regulation of hypothalamic function and many aspects of reproduction in vertebrates. Pollutants such as octylphenol (OP), that mimic the actions of estrogens are therefore candidate endocrine disrupting chemicals. We employed a differential display strategy (RNA-arbitrarily primed PCR) to isolate partial cDNA sequences of neurotransmitter, developmental and disease-related genes that may be regulated by OP or E2 in the turtle hypothalamus. Hatching and year-old male snapping turtles were exposed to 10 ppb waterborne OP or E2 for 17 days. One transcript (421 bp) regulated by 4-OP and E2 was 93% identical to human APLP-2. APLP-2 and the amyloid precursor protein (APP) regulate neuronal differentiation and are also implicated in the genesis of Alzheimer's disease in humans. Northern blot analysis determined that the turtle hypothalamus contains a single APLP-2 transcript of 3.75 kb in length. Exposure to OP upregulated hypothalamic APLP-2 ($p < 0.05$) mRNA levels 2-fold in month old and yearling turtles. E2 did not affect APLP-2 in hatchlings but in contrast stimulated a 2-fold increase ($p < 0.05$) in APLP-2 mRNA levels in yearling males. The protein b-amyloid, a selectively processed peptide derived from APP is also involved in neuronal differentiation, and accumulation of this neurotoxic molecule causes neuronal degeneration in the Alzheimer brain. Therefore, we wished also to determine the effects of estrogens on its expression. Using homology cloning based on known sequences, we isolated a cDNA fragment (474 bp) from turtle brain with 88% identity to human APP. Northern blot analysis determined that a single 3.5kb transcript was expressed in the turtle hypothalamus. Waterborne OP also increased the expression of hypothalamic APP after 35 days of exposure. Our results indicate that low levels of OP are bioactive and can alter the expression of APLP-2 and APP. Given that members of the APP gene family are involved in neuronal development, we hypothesize that OP exposure may disrupt hypothalamic development in young turtles.

RISK ASSESSMENT METHODOLOGIES RESEARCH PROGRAM

Executive Summary:

The general objective of this theme team, since its inception, has been to develop new tools and data for reducing uncertainty and enhancing the scientific basis of the assessment of risks associated with exposure to chemicals. Over the past few years, this team has developed data and methodologies to address specific issues in risk assessment. The current focus of this theme team is to develop data and validate methodologies for performing environmental risk assessments with emphasis on chemical mixtures and interspecies extrapolation.

The proposed efforts will involve the further development of the following tools for enhancing the scientific basis of risk assessments: bioindicators (Greenberg), toxicokinetic models (Law, Mackay, Krishnan), probabilistic models (Solomon) and population dynamic models (Sibley). Greenberg et al. propose to examine mechanisms and molecular changes in response to metal/PAH/oxyPAH mixture exposures with the view of facilitating the application of photosynthetic and respiratory bioindicators in environmental risk assessment. Whereas Law et al. intend to develop physiologically-based toxicokinetic (PBTK) models for risk assessment of cattle exposed to oil and gas chemical mixtures, Mackay et al. propose to develop such models on the basis of structure-property relationships for a number of endocrine-active phthalate esters. Krishnan et al. propose to develop: (i) algorithms for calculating interspecies uncertainty factors for certain species combinations, (ii) a framework for using data on tissue dose to improve the toxic equivalency factor and additivity approaches currently applied in the risk assessment of chemical mixtures, and (iii) a physiological modeling framework for conducting risk assessment of endocrine-active chemical mixtures based on the mechanism of their interaction with hormones. Solomon et al. intend to collect data for facilitating the use of a probabilistic model for the assessment of the ecological risks associated with pharmaceuticals released into surface water via sewage effluent. Research described herein will produce data needed to reduce uncertainty and adequately assess risk associated with the recurrent loading of pharmaceutical mixtures in surface water. Sibley et al. will evaluate the influence of key biological factors that affect demographic endpoints and their application in population growth models. This goal will be accomplished with studies in invertebrates. They also intend to evaluate the probability of extinction, a promising tool that has recently been introduced as a descriptor for assessing the dynamics of stressed populations, to assess the relationship between chemical stressors and population dynamics.

The various research goals will be accomplished by networking among the members of the risk assessment theme team representing seven academic institutions across the country, Alberta Research Council, Environment Canada and Health Canada. This team represents a grouping of experts with complementary abilities in the areas of ecotoxicology, aquatic toxicology, toxicokinetics, mechanistic toxicology, and biologically-based modeling with particular focus on risk assessment applications. Overall, the proposed research work of this multidisciplinary team effort should be useful in improving ecological risk assessments for certain environmentally-relevant pollutant mixtures. In addition, the methodological approaches to be developed during the course of this research program should augment our ability to conduct

scientifically-sound risk assessments for other chemicals and chemical mixtures of national concern.

Highly Trained Personnel:

One of the important features of the CNTC program that deserves special mention is that of the training of high quality personnel. The projects in the Risk Assessment Methodologies Program provided support for 6 students at the graduate level and 7 postdoctoral fellows in fiscal year 2000-2001.

Risk Assessment Methodologies Platform Abstracts:

The use of bioindicators for risk assessment of contaminant mixtures: Synergistic toxicity of the role of reactive oxygen species in ecotoxicology.

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Polycyclic aromatic hydrocarbons (PAHs) and heavy metals are two prevalent classes of persistent contaminants in aquatic and terrestrial environments. Because they often share the same production sources, they frequently co-exist in contaminated environments. However, most toxicological studies of these contaminants are carried out on individual compounds. Few studies have focused on mixtures of the same class of contaminants, and even fewer have investigated the hazards of mixtures of different classes of contaminants such as metals and PAHs. Furthermore, photooxidation of PAHs in the environment will generate many new species of compounds that have not been identified or thoroughly investigated. Therefore, risk assessment has not been performed for environmentally relevant mixtures of these two classes of chemicals. Further, the biochemical mechanisms of the effects of these two classes of chemicals are virtually unknown. We have found that mixture of metals and photomodified PAHs cause synergistic toxicity. The synergistic effects are induced by different modes of action of metals and photomodified PAHs. For instance, an anthracene photoproduct, 1,2 dihydroxyanthouinone, inhibits cytochrome b/f and cytochrome c reductase, and thus, interrupts electron transport in chloroplasts and mitochondria, respectively. Metals use a catalytic mechanisms to generate reactive oxygen species (ROS) by accepting electrons from overly reduced quinones, and succinate or NADH dehydrogenase. Thus, photomodified PAHs inhibit electron transport, while metals harvest the electrons from the blocked bioenergetic membranes to generate high level of ROS. This research has revealed an important catalytic mechanism of metal toxicity, that being apoptosis due to ROS formation. We are examining this problem further by developing new biochemical and molecular indicators of toxicity that can be used as tools in risk assessment. These include a bacterial assay of respiration and development of differential display PCR for *D. magna*, *L. gibba* and rat *Corpus luteum cells*. We are applying these techniques to a wide scope of metal and PAH mixtures.

An interaction-based risk assessment methodology for chemical mixtures.

M. Béliveau, R. Tardif, and K. Krishnan.

Groupe de Recherche en Toxicologie Humaine (TOXHUM), Université de Montréal

The currently used default mixture risk assessment methodologies do not take into account the consequences of potential interactions occurring between chemical components. The occurrence of toxicokinetic and toxicodynamic interactions can result in lower toxicity (antagonism) or greater toxicity (synergism) of mixtures than that expected based on the knowledge of the potency and dose of the constituents. The objectives of this study were: (i) to develop a risk assessment methodology for chemical mixtures that accounts for toxicokinetic interactions among components, and (ii) to apply this methodology to assess the health risk associated with occupational inhalation exposure to air-borne mixtures of dichloromethane, benzene, toluene, ethyl-benzene and m-xylene. The basis of the proposed risk assessment methodology relates to the characterization of the change in tissue dose surrogates [e.g., area-under-the-concentration vs. time curve for parent chemical in tissues (AUC_{tissue}), maximal concentration of parent chemical or metabolite (C_{max}), amount metabolized over a period of time] during mixed exposures using physiologically-based toxicokinetic (PBTK) models. For systemic toxicants, an interaction-based hazard index (THI) was calculated using data on tissue dose of mixtures. An interaction-based hazard index was then calculated for each toxic effect by summing the ratio of AUC_{target tissue} (AUC_{tt}) obtained during mixed exposure (pre-defined mixture) and single exposure (TLV). For the carcinogenic constituents of the mixture, an interaction-based response additivity approach was applied by adding the cancer risk for each constituent, calculated as the product of $q^*_{\text{tissue dose (td)}}$ and AUC_{tt} .

The approaches developed in this study permit for the first time the consideration of the impact of toxicokinetic interactions at a quantitative level in mixture risk assessments for human health and ecosystem.

Fate and toxicity of fluorinated surfactants and 4-Nonylphenol in aquatic microcosms.

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Perfluorinated surfactants (PFSs) are anthropogenic compounds widely used in stain-repellants and fire retardants. Known to be extremely persistent and suspect to bioaccumulate and bioconcentrate, PFSs may present concerns for long-term availability to organisms in aquatic ecosystems. Laboratory toxicity tests were performed with a variety of PFSs ranging from 3 to 10 carbons in length on selected primary and secondary producers. The relative sensitivity of the algae *Chlorella vulgaris* and *Selenastrum capricornutum*, the floating macrophyte *Lemna gibba* and the invertebrates *Daphnia magna* and *D. pulex* was compared for each surfactant. When expressed as molar concentrations, both perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) were the most toxic to all test organisms in lab tests. A parabolic relationship was seen for primary producers when relative inhibition was compared to carbon chain length. No parabolic relationship was observed for either of the *Daphnia* species. *S. capricornutum* was

the most sensitive test species for PFSs. Field studies performed in outdoor mesocosms dosed with PFOA and PFOS showed clear, acute toxicity at the highest dose to *L. gibba*. Since very little work has been done on the fate and toxicological implications of these compounds, this research will be used to support future environmental risk assessment.

Risk Assessment Methodologies Poster Abstracts

Mechanism-based evaluation of the interspecies uncertainty factor used in risk assessments.

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The interspecies uncertainty factor (UF) is used to derive an equivalent exposure dose in one species (e.g., humans) from data in another species (e.g., rat). The UF used in risk assessment has recently been subdivided into two components to account separately for interspecies differences in toxicokinetics (UF_{TK}) and toxicodynamics (UF_{TD}). The former implies that the interspecies difference in toxicity is attributed to differences in tissue dose whereas the latter implies that for the same tissue dose two species would respond differently. The objective of this study was to develop algorithms for estimating the UF for various species combinations involving rat, mouse, and fish (catfish, rainbow trout, fathead minnow). The interspecies extrapolation of doses was performed for one or more of the following chemicals: benzene (BZN), 1,2 dichloroethane (DCE), 1,1,2 trichloroethane (TriCE), 1,1,2,2 tetrachloroethane (TCE), pentachloroethane (PCE), and hexachloroethane (HCE). The approach involved the derivation of mechanistic algorithms from validated physiologically-based toxicokinetic (PBTK) models to calculate interspecies differences in tissue dose for identical exposure conditions. These algorithms related tissue dose to the species-specific mechanistic determinants of toxicokinetics (i.e., body lipid content, tissue:blood partition coefficients, metabolism rate, physiological differences). The difference in tissue dose was quantitated and it corresponded to the toxicokinetic part of the interspecies UF. This factor was used along with the ratio of experimental data on LC_{50} in the appropriate species to determine the contribution of the toxicodynamic aspect of the interspecies UF. For TCE, PCE, and HCE the estimated trout-fathead minnow UF_{TK} were 1.75, 1.09, and 1.88, respectively. The estimated UF_{TD} was 1, 1.6, and 1, respectively. The estimated mouse-rat UF_{TK} was 3 for BZN, while the UF_{TD} was 1. This study represents the first attempt to quantify the chemical-specific interspecies uncertainty factor on the basis of quantitative differences in mechanistic determinants of toxicokinetics in aquatic species and mammals.

Synthesis of Alkyl-phenanthrenes using Directed Metalation-Cross Coupling Methodology

Xiongwei Cai and Victor Snieckus

Department of Chemistry, Queen's University, Kingston, ON

Alkyl-PAH has recently been recognized as being toxic to larval and maturing fish in waterways. Using directed remote metalation-cross coupling strategies developed in our laboratory we have synthesized methyl- and ethyl- phenanthrenes in gram quantities by short (5 - 8 steps) routes in high overall yields (20% - 34%) from readily available starting materials. The route provides alkyl-phenanthrenes as single isomers, many of which are not commercial substances or very expensive, for programs of toxicity testing.

Aquatic macrophytes *Myriophyllum spicatum* and *Myriophyllum sibiricum* exposed to perfluorooctanoic acid (PFOA) in outdoor microcosms.

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Perfluorooctanoic acid (PFOA) is a fluorinated surfactant, recently documented in aquatic systems and wildlife tissues. Fluorinated surfactants have been shown to be persistent under extreme environmental conditions, as well as liver toxicants. Replicate (n=3) 12 m³ enclosed microcosms were dosed with the nominal concentrations of 0.3, 1, 30, and 100 mg/L of PFOA (sodium salt), plus controls. Each microcosm was stocked with 8 individual apical shoots of both *Myriophyllum spicatum* and *Myriophyllum sibiricum* and sampled at regular intervals over a 35-day exposure period. Plants were sampled after 7, 14, 28 and 35 days of exposure and assessed for the somatic endpoints of plant length, root growth, node number, and wet/dry mass and the biochemical endpoints of chlorophyll a/b and carotenoid content. Results showed significant toxicity for most endpoints in both plant species. PFOA concentrations remained constant in the water column, with the exception of the highest concentration, over the course of the fate investigation (35 days), indicating that this compound undergoes little, if any, degradation in aquatic systems. Overall, PFOA does not appear to pose an acute toxic risk to these aquatic macrophytes at current environmental concentrations, but PFOA may increase the risk of chronic toxicity due to the lack of degradation under environmental conditions.

Evaluation of pharmacokinetic interaction between 17 β -estradiol and 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD) in female Sprague-Dawley rats.

C Emond, and K Krishnan, TOXHUM, Université de Montréal, Montréal, Québec, Canada.

Endocrine disruptors (EDs) are substances that interfere with the synthesis, pharmacokinetics or pharmacodynamics of natural hormones responsible for the maintenance of homeostasis, reproduction and development. An interaction between an ED and an endogenous hormone such as 17 β -estradiol (E2) at the pharmacokinetic level can result in a change in the serum concentration of the hormone. The endocrine-disruption potential of certain organochlorine (OC) compounds may be due to their interference with the distribution, metabolism or excretion of E2. Potential EDs such as TCDD can alter the blood concentration profiles of E2 by interfering with its metabolism (e.g., induction or inhibition of E2 metabolism)

and distribution (e.g., competition for receptor binding). The objective of the present study was to evaluate the possible pharmacokinetic interaction between E2 and TCDD in rats. Groups of female Sprague-Dawley rats were administered E2 alone (0.25 mg/kg BW, iv) or along with TCDD (50 or 500 ng/kg BW, iv) and serial sampling of serum was performed at 5, 10, 30, 60, 90, 120 and 180 minutes following administration. Serum E2 levels were quantified by immunoassay. The serum concentration of E2 was significantly greater following co-administration with TCDD. The areas under the serum concentration vs time curve (AUC) at 180 min. were: 37252, 66709, 146948 pmol*L⁻¹*hr whereas the E2 elimination rate constants values were 0.0042, 0.0120, 0.001 for the groups receiving 0, 50 and 500 ng/kg TCDD, respectively. The observed modulation of E2 kinetics in the presence of TCDD could either be due to an interaction at the metabolic level or at the distribution level. To investigate competition for metabolism as the possible mechanism of TCDD/E2 interaction, *in vitro* studies were conducted. The rate of E2 disappearance in the reaction medium was not altered significantly by the presence of TCDD. Overall, TCDD at doses investigated in this study modified the PK profile of E2 and metabolic interaction does not appear to be the major mechanism responsible for the TCDD-induced modulation of E2 kinetics in female Sprague-Dawley rats.

METALS SPECIATION PROGRAM

Executive Summary:

Research over the past 20+ years has demonstrated obvious qualitative links between the speciation of a metal and its general "reactivity", whether it be in a geochemical or a toxicological context. One of the principal goals of the Metal Speciation Theme Team has been to improve these linkages and to put them on a more quantitative footing. The metals chosen for research (e.g., Cd, Zn, Ag) have corresponded closely to those that have been given priority under the Canadian Environmental Protection Act (e.g., on the CEPA Priority Substance Lists) and relevant biological target species have been studied. In addition, the MSTT has focused on metal "species" that are known to exist in natural systems, close to biological interfaces. Carefully controlled laboratory experiments with well-characterized metal speciation are still a common element. During the past year the Metal Speciation Theme Team has investigated the effect of adsorbed humic substances on permeability of intact algal cells towards Cd and Ag (Campbell), Cd and Hg interactions at the human gut epithelial cell interface with both serosal and luminal compartments (Denizeau), Ni binding with sediment organic carbon (Liber), Cd and Cu uptake by plants relative to characteristics of the rhizosphere (Van Rees, Hale), and Cd bioavailability in food (Hale and Black).

Influence of dissolved organic matter (DOM) and reduced sulphur species on the bioavailability of metals at biological surfaces (Campbell) During the past year, the INRS group has carried our experiments to determine the effects of the adsorbed humic substances on the permeability of intact algal cells towards Cd, and has begun to lay the groundwork for similar experiments with Ag. Aquatic humic and fulvic acids have been shown to increase the permeability of biological membranes to lipophilic solutes. In *in vivo* experiments, passive diffusion of fluorescein diacetate (FDA) into the green alga *Selenastrum capricornutum* increased in the presence of Suwannee River humic and fulvic acids at pH 5 (humic > fulvic),

but not at pH 7. The observation of enhanced diffusion at the lower pH is consistent with earlier adsorption measurements, which showed that the association of humic and fulvic acids with the algal surface was greater at pH 5 than at pH 7 (Vigneault et al. 2000). Similar experiments were subsequently designed to follow Cd uptake. Uptake of hydrophilic forms of Cd was unaffected by the presence or absence of (adsorbed) humic and fulvic acids. In both cases Cd uptake obeyed the Free-Ion Model. However, uptake of the lipophilic diethyldithiocarbamate complex, i.e. $\text{Cd}(\text{DDC})_2^0$, was markedly enhanced in the presence of the aquatic humic substances (two-fold increase for the humic acid, 1.5x for the fulvic acid). This demonstration that humic substances can alter the permeability of phytoplankton and model membranes at natural concentrations and pH values has potential implications for the uptake and regulation of toxic and essential solutes by the phytoplankton community. Amiel Boullémant (new MSc student) will be following up this finding.

In parallel, they have extended their earlier silver work (which focused almost exclusively on silver uptake) to consider the influence of the speciation of silver on its toxicity to algae. Previous work had shown that silver uptake is stimulated in the presence of thiosulfate (Fortin and Campbell 2001) and preliminary work suggested that this stimulation of uptake was accompanied by an enhanced toxicity. As a follow-up to this work, *Chlamydomonas reinhardtii* was grown under continuous culture conditions (triplicate turbidostats) in defined inorganic media with and without an inorganic ligand (thiosulfate). Intracellular silver concentrations and a suite of parameters related to algal "health" were monitored over a range of free silver and thiosulfate concentrations. At a constant free silver concentration (~10 nM) and increasing $\text{AgS}_2\text{O}_3^{-1}$ concentrations, toxicity of silver to *C. reinhardtii* is not constant, contrary to what would have been predicted by the FIM. The increased toxicity is presumably related to the ca. 10 fold higher intracellular Ag concentrations reached in the presence of thiosulfate. At the higher intracellular Ag concentrations encountered in the presence of thiosulfate, growth rates and photosynthetic rates become uncoupled. These experiments remain to be repeated to confirm the results and tests using another algal species, *Selenastrum capricornutum*, will confirm the generality of the algal response to Ag in the presence and absence of thiosulfate.

Membrane transport processes and subcellular interactions that influence metal toxicity (Denizeau and Jumarie): The broad objective of identifying the key steps modulating Cd bioavailability in humans following oral exposure was approached by identifying the major intracellular pool(s) for Cd once transported into the intestinal cells. The use of fluorescent dyes affords a unique opportunity to improve our investigation of metal interactions with sub-cellular compartments. The confocal microscope at TOXEN has been upgraded with a cooled digital camera and the two dyes APTRA and BTC-5N, highly specific to Cd^{2+} , were used in this study. The optimal concentration for these dyes has been established to be 3 μM and a dissociation constant (K_d) of 1 μM and 0.1 μM has been determined for APTRA and BTC-5N, respectively. Since the living cells contain relatively high levels of Cl^- (~10 mM), thus reducing the level of the free Cd^{2+} available to react with the dyes, a significant signal-over-noise ratio had to be verified in the presence of an excess of Cl^- . Results obtained with a FACS VANTAGE flow cytometer confirmed that: i) a 30 min incubation in 3 μM of either of the two dyes does not affect cell viability; ii) significant variations in fluorescence are observed with Cd preloaded cells

compared to control samples (only pretreated with the dye). Preliminary data obtained with adhesive cells and the CCD imaging system show significant increases in intracellular BTC-5N fluorescence upon Cd addition in the incubation medium. A very rapid response occurs within seconds and appears as intracellular spots with blurrings. Experiments are underway to identify these intracellular compartments using co-localisation software.

In recent years, studies at UQAM have focussed on membrane transport of Cd as a function of metal speciation in human intestinal cells (Caco-2 cells). However, the balance between the influx and efflux systems present in the cell membrane is crucial in determining the intracellular levels of metals and their toxic effects. The UQAM researchers now want to extend their work to efflux pumps (P1-type ATPases) in rainbow trout hepatocytes as systems playing a key role in the health of ecosystem organisms. This orientation is also in line with the recommendation of the EAC that, within the context of CNTC, we should put the emphasis on ecosystem health rather than on human health.

Bioavailability of metals to plants and their mammalian consumers: studies of Cd and Cu (Hale and Black): The long-term objective of this project is to enable the prediction of metal transfer from soils to mammals (wildlife or humans), via dietary intake of plants. Towards that end, we are studying the relationship between metal free-ion concentration in soil and plant uptake of metals, and the relationship between metal speciation in diet and absorption by intestinal cells. Previous work in our laboratory, as well as other studies, suggest that the Free Ion Activity Model of metal bioavailability does not describe Cd uptake by plants, in the presence of several inorganic and organic ligands that would be expected to occur in soil solution. The Biotic Ligand Model of metal bioavailability, which is related to FIAM, treats the biological membrane as an organic ligand which is in competition with other ligands for the metals, and so determines the a conditional stability constant for the root-metal complex. We are pursuing the BLM for metal bioavailability for Cu; the solution chemistry for the first experiments is being determined using MINTEQA2 for minimal hydroponic solutions, with citrate and NTA as additional ligands.

An *in vitro* digestion technique for metal-containing lettuce and grain has been developed, by modifying a technique developed for estimating the bioavailable fraction of metal in ingested soils. Lettuce with Cd incorporated during growth was digested using this method, and gastric and intestinal digestion phases were analyzed for Cd. At the end of the gastric digestion phase, more Cd was found in the filtrate fraction than the solid fraction; after the intestinal phase, at higher pH, the Cd appears to have reassociated with the solid fraction relative to the concentration found in the filtrate. This standard operating procedure will be used in the next experiments to measure the transfer of Cd from digested food to cultured intestinal epithelial cells. This extension to cultured intestinal epithelial cells constitutes a new link between UQAM and the University of Guelph, one that shall be exploited in the next year through training opportunities .

The significance of organic carbon in modifying metal bioavailability in freshwater sediments (Liber): The primary objective of this project is to evaluate the significance of different forms/sources of organic carbon as binding phases for nickel under both water-only and

sediment conditions. Three different sources of DOC were obtained or isolated, including Suwannee River humic and fulvic acids, peat humic and fulvic acids as well as peat hydrophilic DOC (aqueous), and Little Bear Lake humic and fulvic acids isolated from actual sediment pore water. Peat DOC appears to have a higher ratio of fulvic to humic acids, and a greater percentage of total hydrophobic acids, than the other two sources of DOC. These sources of DOC were used in water-only, 48 h (without feeding) Ni toxicity/bioavailability testing of *Hyalella azteca*; these tests demonstrated no clear effect of DOC on nickel toxicity, or any discernable trends in the 48-h LC50 data. It appears that while DOC is known to complex nickel, the molar concentrations of nickel required to produce a 48-h LC50 for *H. azteca* are greater than can be significantly complexed by the DOC. Use of the MSTT Ion Exchange Technique, as well as MINTEQA2, to determine the free Ni^{2+} in solution has demonstrated that at acute concentrations of Ni, >80% of the metal is Ni^{2+} , regardless of the DOC type. This finding confirms the toxicity testing results, and suggests that DOC may be more important as a modifier of bioavailability in chronic exposures.

Production of low molecular weight organic acids in plant rhizospheres and relationship to cadmium uptake (Van Rees): The objective of this project is to study the association between the gene for cadmium (Cd) uptake in durum wheat and the production of low molecular weight organic acids (LMWOAs) found in the rhizosphere of two random populations of durum wheat that segregate for Cd uptake. Sixty four lines (50% are high accumulators and 50% are low accumulators) of durum wheat were grown in a pot experiment in order to determine LMWOAs concentrations in the rhizosphere and the relationship to the marker linked to the Cd uptake gene *cdu-1*. Plant Cd concentrations showed a bimodal distribution and were well linked to the two marker groups (no gene and gene present) suggesting that the gene controls Cd concentration in the plant tissues. With respect to the LMWOAs, only 12 lines have the analysis completed, however, there is a significant relationship between the two marker groups and LMWOA production. Lines with the marker produced high levels of LMWOAs while lines without the gene produced lower levels of LMWOAs which suggests that the gene controlling Cd uptake may also function in the production of LMWOAs from the root systems.

In addition to conducting studies that lead to successful graduate degrees and publications in peer-reviewed journals, the activities of the Metals Speciation Theme Team have supported and will continue to support four of the five objectives of the CNTC, as described in the Network's Strategic Operating Plan:

- Efficient and coordinated understanding of toxic substances
- Focus of MSTT is metal bioavailability, key to predicting environmental exposure of receptors to metals
- Communication among toxicology centres in the areas of research and training
- In May 2000, Lorne Doig visited the INRS-Eau laboratories to learn how to determine free Ni^{2+} concentrations in his Ni-DOM systems. This ion exchange technique, developed with CNTC funding, has now been transferred to two of the MSTT participating laboratories (B. Hale, Guelph; K. Liber, Saskatoon)

- Canadian Institutes for Health Research grant to Hale, Black and Coomber (Dec 2000-Nov 2003) was developed from seed projects funded by CNTC; this interdisciplinary research project will cross-train students in plant and mammalian sciences, and will expose the trainees to ecological risk assessment
- Joint co-operative research between CNTC and federal agencies/industry
- Kodak Canada/NSERC (Campbell)
- Cameco/Sask. Environmental Research Management/CNTC (Liber)
- MITE Research Network (Campbell, Hale, Edwards)
- Develop and maintain high standards of scientific research in toxicology
- Guidelines document on metals experiments for CNTC website is available on the CNTC web site

Highly Trained Personnel:

One of the important features of the CNTC program that deserves special mention is that of the training of high quality personnel. The projects in the Metals Speciation Program provided support for 10 students at the graduate level and 1 postdoctoral fellow in fiscal year 2000-2001.

Metal Speciation Platform Abstracts

Influence of thiosulfate and natural organic matter on metal uptake and toxicity to freshwater algae.

Véronique Hiriart, Claude Fortin, Bernard Vigneault and Peter G.C. Campbell.
Université du Québec, INRS-Eau, Sainte-Foy, PQ.

Silver toxicity towards the freshwater green alga *Chlamydomonas reinhardtii* is being investigated under continuous culture conditions by the use of turbidostats in defined inorganic media with and without an inorganic ligand (thiosulfate). In the absence of thiosulfate, algal growth was inhibited by 50% at a free Ag^+ concentration of 16 nM Ag^+ (EC_{50}). Growth rates calculated for the first 12 h after a Ag treatment spike were significantly lower than during the following 12 h, indicating some adaptability of the algae to Ag exposures. However, these differences were absent at the higher Ag exposures, suggesting some threshold of tolerance where the alga's acclimation abilities become overwhelmed. Photosynthesis was reduced in the presence of Ag and could be inhibited by as much as 64%. In the presence of the inorganic ligand, thiosulfate ($\text{S}_2\text{O}_3^{2-}$), silver uptake was stimulated four-fold, even though the free Ag^+ concentration was kept constant in the two experiments (Environ.Sci. Technol. in press). This effect was further enhanced when the algal cells were sulfate-starved before exposure. We interpret this result as evidence for the <accidental transport> of $\text{Ag-S}_2\text{O}_3^{-1}$ via the $\text{SO}_4^{2-}/\text{S}_2\text{O}_3^{2-}$ transport system present in the plasma membrane. Preliminary work suggests that this stimulation of metal uptake is accompanied by a higher toxicity: algae exposed to 30 nM Ag^+ with thiosulfate showed no positive growth, whereas algae exposed to 30 nM Ag^+ only showed reduced (43% of control) but positive growth.

In parallel with this work on the Ag-thiosulfate system, we studied the influence of natural dissolved organic matter (DOM) on the bioavailability of metals. Uptake of the lipophilic

cadmium-diethyldithiocarbamate complex, i.e. $\text{Cd}(\text{DDC})_2^0$, was markedly enhanced in the presence of the aquatic humic substances (two-fold increase for the humic acid, 1.5x for the fulvic acid). This demonstration that humic substances can alter the permeability of phytoplankton membranes at natural concentrations and pH values has potential implications for the uptake and regulation of toxic and essential metals by the phytoplankton community.

Subcellular localization of Cd using CCD imaging system and confocal microscopy

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The broad objective of identifying the key steps modulating Cd bioavailability in humans following oral exposure was approached by identifying the major intracellular pool(s) for Cd once transported into the intestinal cells. Identification of the sub-cellular binding/accumulation sites and evaluation of how other metals may modulate Cd interactions with them are needed for a better understanding of parameters modulating the intestinal absorption of metals as well as synergistic toxic effects, especially in view of metal absorption from mixtures.

The use of fluorescent dyes afforded a unique opportunity to improve our investigation of metal interactions with sub-cellular compartments. The two dyes APTRA and BTC-5N, highly specific to Cd^{2+} , were used in this study. We also studied the response of the dyes to levels of Cd^{2+} as a function of Cd speciation using chloride or nitrate media. The living cells contain relatively high levels of Cl^- (~ 10 mM), which significantly decreases the level of the free Cd^{2+} available to react with the dyes. However, our results show that despite the high degree of Cd complexation by Cl^- , we were able to get significant signal-over noise fluorescence ratios using non cytotoxic metal levels.

Results obtained with a FACS VANTAGE flow cytometer confirmed that: i) a 30 min incubation in 3 μM of either of the two dyes does not affect cell viability; ii) significant variations in fluorescence are observed with Cd preloaded cells compared to control samples (only pretreated with the dye). Preliminary data obtained with adhesive cells and the CCD imaging system showed significant variations in APTRA fluorescence upon Cd addition in the incubation medium. A very rapid response occurred within seconds and appeared as intracellular spots with blurrings. Further experiments are now in progress to identify these intracellular compartments using co-localisation software.

The significance of dissolved organic carbon in modifying nickel speciation and bioavailability to the amphipod *Hyaella azteca*.

L. Doig and K. Liber

Toxicology Centre, University of Saskatchewan, Saskatoon, SK.

The effect of dissolved organic matter (DOM) on nickel bioavailability in sediment and hence nickel toxicity to aquatic organisms is largely unstudied. For this research, three different sources of DOM were obtained or isolated. These included: Suwannee River humic and fulvic acids (obtained from the International Humic Substances Society, IHSS); peat humic and fulvic

acids, as well as whole peat extract; and Little Bear Lake (LBL) humic and fulvic acids isolated from LBL sediment pore water. A series of nickel toxicity experiments were conducted using DOM from the various sources and fractions to evaluate effects on nickel bioavailability and toxicity to *Hyalella azteca* in 48-h tests. It was found that there were no discernable trends in the 48-h LC50 data. Using a miniaturized ion exchange technique (IET), it was shown that at 5 mg/L total Ni (a concentration just below the average 48-h LC50) and 10 mg/L DOC (of various sources and fractions) the free nickel ion concentration was only slightly altered in comparison to a solution lacking DOM. While it is known that nickel will complex with organic matter, the nickel concentrations required for acute toxicity were likely greater than could be significantly complexed by the DOM. Further IET work demonstrated that, at lower molar concentrations, a greater relative proportion of the free nickel was able to complex with DOM. Therefore, DOM likely plays a much greater role in reducing nickel bioavailability under lower, chronic exposure situations. Modeling (MINTEQA2) of the nickel test solutions using EDTA and citrate as ligands (30 mg/L DOM in reconstituted water) generally support the findings of the toxicity testing and IET. They suggest that while nickel speciation can be significantly affected by organic ligands at low total nickel concentrations (i.e., below the 48-h LC50), higher concentrations of total nickel remain largely in the free ion form and hence bioavailable.

Cadmium accumulation in durum wheat.

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Cadmium (Cd) level in wheat has drawn extensive attention in the world because it has the potential to contaminate food and damage human health and the ecosystem. The Cd accumulation has been found to vary among cultivars of durum wheat, and it is hypothesized that low molecular weight organic acids (LMWOAs) produced at the rhizosphere of plants may importantly affect the uptake of Cd in durum wheat. A molecular marker, sequence characterized amplified region (SCAR) was used as an effective method to screen the Cd uptake in durum wheat. The objective of this study was to investigate the relationship between low molecular weight organic acid (LMWOA) production in the rhizosphere of durum wheat and the gene for low cadmium uptake. The 62 lines and their parent (Stewart 63/Biodur) were grown in pot-culture under growth chamber conditions. Biodur is a low cadmium uptake cultivar while Stewart 63 is high cadmium uptake cultivar. The experiment showed that there was a good relationship between the marker and cadmium levels in the recombinant inbred population. Total Cd amount in the marker group was significantly higher than that for the no marker group. The results showed that one gene controlled Cd uptake in durum wheat. The lines showing the Cd marker tended to accumulate larger amounts of Cd and produce higher amounts LMWOAs than the low Cd uptake lines, but the total amount of LMWOAs in the marker group was not significantly higher than that for the no marker group.

Metals Speciation Poster Presentations

***In vitro* Method for Determining Transfer of Metals from Food to Mammals**

D. Boyle, D. Chan, W. Black and B. Hale
University of Guelph

The development of in vitro methods to estimate the absorbable fraction of metals in foods and soils would be a valuable addition to risk assessments for human exposure to metals. Digestion can be stimulated outside of the human body using enzymes and acid preparations to mimic gastric and intestinal processes. Absorption of metals from the product of the intestinal processes is the final step in such a method. As part of a project to develop the absorption step, we needed to establish a protocol for digesting food materials containing metals. Specifically, we needed to determine whether freezing the food prior to digestion influenced the recovery of cadmium that had been incorporated into the leaves during growth. Lettuce was grown hydroponically with cadmium at four concentrations (0, 5, 50 and 500 µg/L), resulting in plant material with cadmium concentrations ranging between 0.03 and 80 mg/kg. This material was then subjected to gastric digestion followed by intestinal digestion, and then the solid and filtrate material from each of these phases was analyzed for cadmium concentrations; either fresh or frozen (-80°C) material was digested. In all experiments the amount of Cd in the filtrate fractions was correlated with the amount of Cd in the lettuce; approximately 100 fold more cadmium was associated with the solid phase than with the filtrate. Freezing influenced the extraction process, in that the Ph was more variable during the gastric digestion phase for the fresh tissue; we attributed this to the lysing of cells during the digestion that would have pre-occurred in the frozen tissue. The cadmium concentrations in the frozen versus fresh extractions were similar for the filtrate, but freezing appeared to cause considerably more variability in the Cd concentration in the solid fraction, relative to the non frozen samples. The source of this variability is uncertain; we conclude that the material must be fresh for assessment of absorbable fraction of the Cd contained therein.

The Application of the Biotic Ligand Model to Plant-Metal Interactions

P. Cypas, D. Boyle and B. Hale
University of Guelph

The theoretical basis of the Biotic Ligand Model (BLM) is presented as a useful complement to the Free Ion Activity Model (FIAM) in predicting metal binding and uptake. By merging these models and incorporating experimentally derived thermodynamic and kinetic data, an adaptable model for predicting copper uptake will be created. Thermodynamic conditional stability constants for surface-site binding reactions for the roots of Durum Wheat (*Triticum turgidum*) will be determined as well as the interactive effects imposed by the presence of citrate, nitriloacetic acid (NTA) and various cations on copper binding. The objective is to create a model that is easily adapted for use in natural environments - its success gauged by the accuracy of copper uptake prediction in the presence of plant exudates and low molecular weight metabolic compounds.

Life with MIFE: Is it for you?

K.C.J. Van Rees and R.E. Farrell

Department of Soil Science, University of Saskatchewan

MIFE, not to be confused with MITE, is a method known as the Microelectrode Ion Flux Estimation system. The MIFE apparatus is designed to determine ion fluxes next to biological surfaces and the focus of our work will be to quantify metal fluxes next to biological surfaces. The goal of our project is to understand cadmium fluxes next to root surfaces of high and low cadmium accumulating cultivars of durum wheat and how and what mechanisms may be responsible for differences in flux between the two cultivars. We would, however, like to develop this instrument so that other researchers within CNTC have the opportunity to collaborate with our lab on similar projects or use this instrument for other research. Thus the objective of this poster is to facilitate discussion with other researchers within CNTC to see if future collaborations can be forged around this novel technique.