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**ECOTOXICOLOGICAL IMPACTS OF
ZEBRA MUSSELS, *DREISSENA POLYMORPHA*,
A NEW FOOD SOURCE FOR LESSER SCAUP, *AYTHIA AFFINIS***

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June 1996

A Thesis Submitted to
the Faculty of Graduate Studies and Research
in Partial Fulfilment of the Requirements for
the Degree of Doctor of Philosophy

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0-612-30403-5

ABSTRACT

Ecotoxicological impacts of zebra mussels, *Dreissena polymorpha*, a new food source for lesser scaup, *Aythya affinis*.

Catherine Tessier, Department of Natural Resource Sciences, McGill University

Ph. D., June 1996.

Zebra mussels (*Dreissena polymorpha*) have invaded a great proportion of the waters of the eastern part of North America. This mollusk may be a useful sentinel species for bioaccumulation of heavy metals and organic contaminants in aquatic ecosystems. The zebra mussel's capacity to bioaccumulate cadmium at environmentally relevant exposure and to sequester metals in metal-binding proteins, metallothioneins, was investigated. Elevated (relative to control) concentrations of Cd^{2+} were detected in *Dreissena* exposed to $\geq 2 \mu\text{g Cd/liter}$ suggesting that zebra mussels cannot regulate Cd^{2+} at trace exposure concentration. More than 85% of the measured Cd^{2+} was bound to metallothioneins.

Lesser scaup (*Aythya affinis*) was assessed as a model species for potential impacts of zebra mussels on higher trophic levels. The feasibility of incubating and rearing scaup in semi-natural conditions was evaluated. A series

of biomarkers was assessed in 3 groups of scaup fed a contaminant-free diet or diets containing zebra mussels from the St-Lawrence River or Lake Erie.

Lesser scaup proved to be a suitable species to raise in captivity providing daily water misting through out incubation and strict hygiene conditions during brooding. A hatching success of 89% was achieved. Appropriate housing, feeding and bathing conditions yielded low (3%) post-hatching mortality.

Phagocytosis and respiratory burst activities of heterophils of scaup were suppressed after 6 weeks of feeding on zebra mussels, compared to the control group. These two heterophilic functions were negatively correlated with the incidence of pododermatitis (bacterial feet infection), suggesting a suppression of the non specific immunity. Increased liver/body mass ratio and decreased hepatic vitamin A (retinol and retinyl palmitate) concentrations were observed in scaups fed zebra mussels. Lipid accumulation and glycogen overload were found in the livers of scaups fed mussels from of the St-Lawrence River and the Lake Erie groups, respectively. These immunological, biochemical and histopathological biomarkers show promise for monitoring "early" injury and may help in the understanding of health impairment of different species of waterfowl exposed to xenobiotics via contaminated food sources.

RÉSUMÉ

Impacts écotoxicologiques de la moule zébrée, *Dreissena polymorpha*, comme source de nourriture pour le petit morillon, *Aythya affinis*.

Catherine Tessier, Département des sciences des ressources naturelles,
Université McGill, Ph.D., Juin 1996.

La moule zébrée (*Dreissena polymorpha*) a colonisé une grande proportion des eaux de la côte est de l'Amérique du Nord. Ce mollusque pourrait servir d'espèce sentinelle pour la bioaccumulation des métaux lourds et des contaminants organiques dans les écosystèmes aquatiques. La capacité de la moule zébrée de bioaccumuler le cadmium à des doses représentatives du milieu naturel et de sequestrer ce métal dans les métallothionéines a été examinée.

Des concentrations élevées (comparativement au groupe contrôle) de cadmium chez *Dreissena* ont été détectées à des doses de 2 µg Cd/litre et plus. Ces résultats démontrent que la moule zébrée ne peut régulariser l'ion Cd²⁺ à de faibles concentrations. Plus de 85% du Cd²⁺ mesuré était complexé aux métallothionéines.

Le petit morillon (*Aythya affinis*) a été évalué comme espèce modèle concernant les impacts potentiels de la moule zébrée sur les niveaux trophiques

supérieurs. La faisabilité d'incuber et d'élever les oisillons dans des conditions semies-naturelles a été examinée. Une série de biomarqueurs a aussi été évaluée à l'intérieur de 3 groupes de morillons se nourrissant d'une diète exempte de contaminants, de moules zébrées provenant du fleuve St-Laurent, ou du lac Érie.

Le petit morillon semble être une espèce appropriée pour l'élevage en captivité pourvu que des conditions d'humidité à l'aide d'une légère bruine d'eau soit vaporisé lors de l'incubation et que des conditions strictes d'hygiène soient respectées. Un succès d'éclosion de 89% a été obtenu. Le bas taux (3%) de la mortalité post-éclosion est principalement dû aux conditions adéquates des infrastructures servant à loger les oiseaux (volière et bassins d'eau) ainsi qu'à une nourriture spécialement formulée pour les canards plongeurs.

L'activité phagocytaire et la formation de peroxydes chez les cellules hétérophiles ont été partiellement inhibées après 6 semaines chez les 2 groupes de morillons se nourrissant de moules zébrées comparativement au groupe contrôle et étaient négativement corrélées avec l'incidence de pododermite (infection bactérienne aux pattes). Une augmentation du ratio foie/ masse corporelle et une diminution hépatique des concentrations de vitamine A (rétinole et palmitate de rétinole) ont été observées chez les petits morillons se nourrissant de *Dreissena*. Une accumulation hépatique de lipide et un surcharge en glycogène ont été diagnostiquées chez les canards du groupe du fleuve St-Laurent et du Lac Érié, respectivement. Ces biomarqueurs immunotoxiques, biochimiques et histopathologiques semblent prometteurs pour le dépistage

précoce d'altérations néfastes au niveau cellulaire et peut nous aider à comprendre les problèmes de santé potentiels des différentes espèces de sauvagine exposées à des sources de xénobiotiques via leur régime alimentaire.

Dedicated To Félix

For His Love, Support, And Encouragement

STATEMENT FROM THESIS OFFICE

In accordance with the regulation of the Faculty of Graduate Studies and Research of McGill University, the following statement excerpted from the Guidelines for Thesis Preparation (McGill University, 1995) is included:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

*If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.*

*The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.*

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

*In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.*

ADVANCEMENT OF SCHOLARLY KNOWLEDGE

1. Contribution to Advancement of Knowledge

The following are original contributions to knowledge by this thesis:

(1). The development of an improved incubation and brooding method for studying and raising diving ducks in captivity using lesser scaup as a model species. This will foster more realistic studies of the ecotoxicological impacts of chemicals which could be released to the environment.

(2). The development of a new methodology to evaluate immune related functions in bird heterophils using flow cytometry and, for the first time, the assessment of heterophilic phagocytic activity and oxidative burst in waterfowl contaminated from naturally ingested food items.

(3). The assessment of a series of physiological and biochemical biomarkers to test their validity as tools for field experimentation with wildlife species.

(4). The evaluation of the benefits of using a series of biomarkers, as opposed to one, in assessing possible health impacts to lesser scaup feeding on a new and contaminated food source, the zebra mussel (*Dreissena polymorpha*).

(5). Metallothioneins have for the first time been detected and investigated in zebra mussels. It was demonstrated that zebra mussels cannot regulate cadmium at low chronic exposure. Moreover, chronic exposure to cadmium at present "natural" levels did not produce a genetic induction of metallothionein as is generally observed in specimens exposed to high concentration of metals.

2. Research Publications from thesis work in Refereed Scientific Journals

- (1). Tessier, C. and J.S. Blais. 1996. Determination of cadmium-metallothioneins in zebra mussels exposed to subchronic concentration of Cd^{2+} . *Ecotoxicol. Environ. Safety*. In press.

3. Manuscripts submitted to refereed scientific journals and proceedings.

- (2). Tessier, C., I. Ritchie, and D. Leclair. 1996. Artificial incubation and brooding of lesser scaup, *Aythya affinis*. Submitted to *J. Wildl. Manag.*
- (3). Tessier, C., D. Flipo, J.S. Blais, and M. Fournier. 1996. Developmental methodology for the evaluation of immune functions in lesser scaup feeding on zebra mussel from polluted water. Submitted to *Environ. Toxicol. Chem.*
- (4). Tessier, C. E. Mazak, S. Trudeau, G. Fox, S. Laird, F. Whoriskey, and J.S. Blais. 1996. Ecotoxicological impacts of zebra mussels (*Dreissena polymorpha*) as a food source for lesser scaups (*Aythya affinis*). Submitted to *Environ. Toxicol. Chem.*
- (5). Tessier, G. Chamberlan, C. E. Mazak, G. Fox, D. Flipo, and J.S. Blais. 1996. Ecotoxicological impacts of zebra mussels (*Dreissena polymorpha*) as a food source for lesser scaups (*Aythya affinis*). *Proceedings of International Symposium on Biological Markers of Pollution*, pp. 379-387, September 21-22, 1995, ANPP, Chinon, France.

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS

The first author was in charge of experimental design, laboratory and field set-up, experimental execution and management, data collection and statistical analyses, preparation of manuscripts for publication and response to the reviewers' comments.

Dr. J.S. Blais was responsible for part of the research funding, administration, and metallothionein methodology development and general guidance as a co-supervisor.

Dr. F. Whoriskey contributed in general guidance through research supervision and funding, technical assistance in zebra mussel sampling and provided extensive comments on all manuscripts.

I. Richie, technician at the Avian Science and Conservation Centre, provided valuable help in incubation of lesser scaup and comments on manuscript (2).

D. Leclair, veterinarian, provided help in brooding and raising lesser scaup, as well as a veterinary follow-up until the ducks reached fledging. He provided comments on manuscript (2).

E. Mazak provided assistance in zebra mussel and diving duck sampling in Lake Erie, coordination and technical help for organochlorine contaminant analysis.

D. Flipo developed part of the methodology for immune responses and contributed to the writing of manuscript (3) under the supervision of M. Fournier.

S. Trudeau, Canadian Wildlife Service, was responsible for the optimization of biomarker methodology and supervision of biomarker determinations reported in manuscript (4).

G. Fox, Canadian Wildlife Service, provided valuable comments and guidelines through out the project and comments on manuscript (4).

S. Laird, Médecine vétérinaire, Université de Montréal, was responsible for the histopathological examination of lesser scaup organs reported in manuscript (4).

The project was, from its inception, perceived to be a multidisciplinary effort. No one individual could have mastered all these skills without the contributions of the collaborators.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Fred Whoriskey and Dr. Jean-Simon Blais, supervisor and co-supervisor of my Ph. D., for their support, advice, encouragement and assistance.

I gratefully acknowledge the advice and commentaries on my work from Guy Chamberland, Rodger Titman, Daniel Leclair, Ian Richie, Ed Mazak, Denis Flipo, Kim High, Suzanne Trudeau and Glen Fox.

I also thank D. Bird and J.R. Bider for use of facilities at the Avian Science and Conservation Centre and at the Ecomuseum, Ste. Anne de Bellevue. A special thanks goes to R.G. Clark and his team (Canadian Wildlife Service, Saskatoon) for their help in collecting scaup eggs.

The author gratefully acknowledges the financial support from the National Engineering Council, the Wildlife Toxicology Fund, the Great Lakes University Research Fund (Environment Canada) and personal scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR).

My deepest gratitude goes to my family and to Félix Blackburn who supported and encouraged me throughout my Ph. D. This thesis would not have been possible without their help and love.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 A new predator-prey relationship

The arrival of zebra mussels (*Dreissena polymorpha*) in North America in 1986-87 has brought fundamental changes to our aquatic ecosystems. It has provided the opportunity to study the impacts of an exotic invader taking over a new ecological niche and disturbing native aquatic communities. Many researchers had insisted on the need to understand the impacts of the zebra mussel on higher trophic levels. As more and more diving ducks have been observed feeding intensively on zebra mussels in the Great Lakes during their migration, and given that previous work demonstrated that filter feeding zebra mussels accumulated a great deal of anthropogenic xenobiotics, the need to study the possible impacts of this mussel on the health of diving ducks has become critical.

1.2 The use of field biomarkers

Biomarkers have long been used in human medicine to aid in the diagnosis of illness. More recently biomarkers have been used to assess health impacts in animals exposed to contaminant stress. These biomarkers have generally been tested under laboratory conditions with domestic strains of animals, following a single acute exposure, in a controlled environment.

However, for these biomarkers to be of ecological use they should also be evaluated in natural (complex and variable) environments with wildlife species exposed to a multitude of contaminants. Several factors including a scientific preference for reductionist approaches, financial considerations, and low cost/benefit ratios (in terms of papers published per unit of cost and time) have resulted in very few researchers focussing their attention on systematic approaches using biomarkers to predict the health of populations. More research is needed to understand the impacts of sublethal contaminant exposure and its implications in relation to the general health of the organism. Thus, "early" biomarker responses may well one day be powerful predictive tools for management and conservation purposes.

1.3 Zebra mussel as a sentinel species for metallic contamination

The zebra mussel has been used as a sentinel species in Europe due to the fact that it is widely distributed geographically, sedentary, and easy to collect. Also, *Dreissena* has a relatively high filtering capacity which makes it valuable as a sentinel species reflecting the toxic burden of aquatic ecosystems. This mollusk can withstand relatively high metal concentrations and appears to be useful to monitor heavy metal dynamics in rivers and in lakes. However, the sequestration dynamics of heavy metals such as cadmium by metallothioneins are unknown for this mollusk.

1.4 Objectives

My general goals were to: a) assess and characterize the dynamics of contaminants (halogenated hydrocarbons) in Lake Erie and St. Lawrence River food webs as modified by the zebra mussel and one of its predators, the lesser scaup, b) evaluate the ecotoxicological impacts of contaminants transferred from zebra mussels to lesser scaup, using a series of physiological, immunological, histopathological and biochemical markers, and c) assess the utility of using a battery of biomarkers as opposed to just one to evaluate the general health condition of lesser scaup.

The more specific goals were to determine and compare: a) the porphyrin profile b) the ethoxyresorufin-o-deethylase (EROD) activity, c) the vitamin A concentrations d) the histopathological alterations e) the relative mass of different organs and f) immunological functions in lesser scaup fed a control diet or zebra mussels from the St. Lawrence River or Lake Erie, under semi-natural conditions. The control group represents the situation before the arrival of *D. polymorpha* when freshwater mussels constituted a very small portion of lesser scaup diet (Longcore and Cornwell, 1964; Rogers and Korschgen, 1966).

Furthermore, we evaluated the potential of zebra mussels to act as a sentinel species of metal contamination. The objectives were to determine if metallothionein (MT), a metal-induced protein biomarker, was present in zebra mussel tissues and to see if MTs were induced during exposure to environmentally relevant concentrations of cadmium.

This thesis is based on four published/submitted research papers. The results are presented in manuscript format. Each chapter is relatively independent from the others, but all are integral parts of the thesis. A literature review is presented in Chapter 2. It summarizes the current state of knowledge in the development and application of the different biomarkers used in this study, the organic contamination found in zebra mussels and aquatic bird species, and emphasizes the need for a reliable series of sensitive biomarkers to assess health impacts on wildlife. Chapter 3 deals with the logistic approach and feasibility of raising diving ducks in captivity. A pilot experiment was necessary in the first year of my Ph.D. to optimize conditions for the lesser scaup model raised under semi-natural environmental conditions. In Chapter 4, the development and application of immunological responses as “early warning” biomarkers is examined. In Chapter 5, a series of biochemical, physiological and histological biomarkers was assessed to determine the possible ecotoxicological impacts of zebra mussels on lesser scaup health. In Chapter 6, I examined the use of metallothionein as a biomarker in zebra mussels. This mollusk is known not only to accumulate organic contaminants but also large amounts of heavy metals which could be transferred to subsequent trophic levels. The thesis closes with a summary and a conclusion (chapter 7).

LITERATURE REVIEW

2.1 The invasion of an exotic species

The Great Lakes watershed has recently been invaded by an exotic species, the zebra mussel (*Dreissena polymorpha*). These mussels were introduced to Canada as larvae via ships' ballast water and were first reported in Lake St. Clair in 1988 (Hebert *et al.*, 1989). Given its great dispersal potential and high reproductive rate, the zebra mussel is predicted to invade a high proportion of North America's freshwater (Strayer, 1991). The distribution of this mollusk is now broader than was first anticipated (New York Sea Grant, 1996) (Fig. 2.1). For example, rivers were not expected to be significantly colonized because high currents would prevent zebra mussel larvae attachment. However, the zebra mussels are now present in large numbers in several high velocity rivers. They are also in southern North American locations well beyond what was predicted based on their known thermal biology (New York Sea Grant, 1996).

Zebra mussels are now an important part of the benthic biomass of the Eastern Great Lakes and the St. Lawrence River (Holland, 1993; Nicholls and Hopkins, 1993; Mellina and Rasmussen, 1994; Nalepa, 1994). Zebra mussel impacts such as increased water transparency, reduced turbidity and reduction in abundances of phytoplankton, zooplankton and unionid mollusks have already

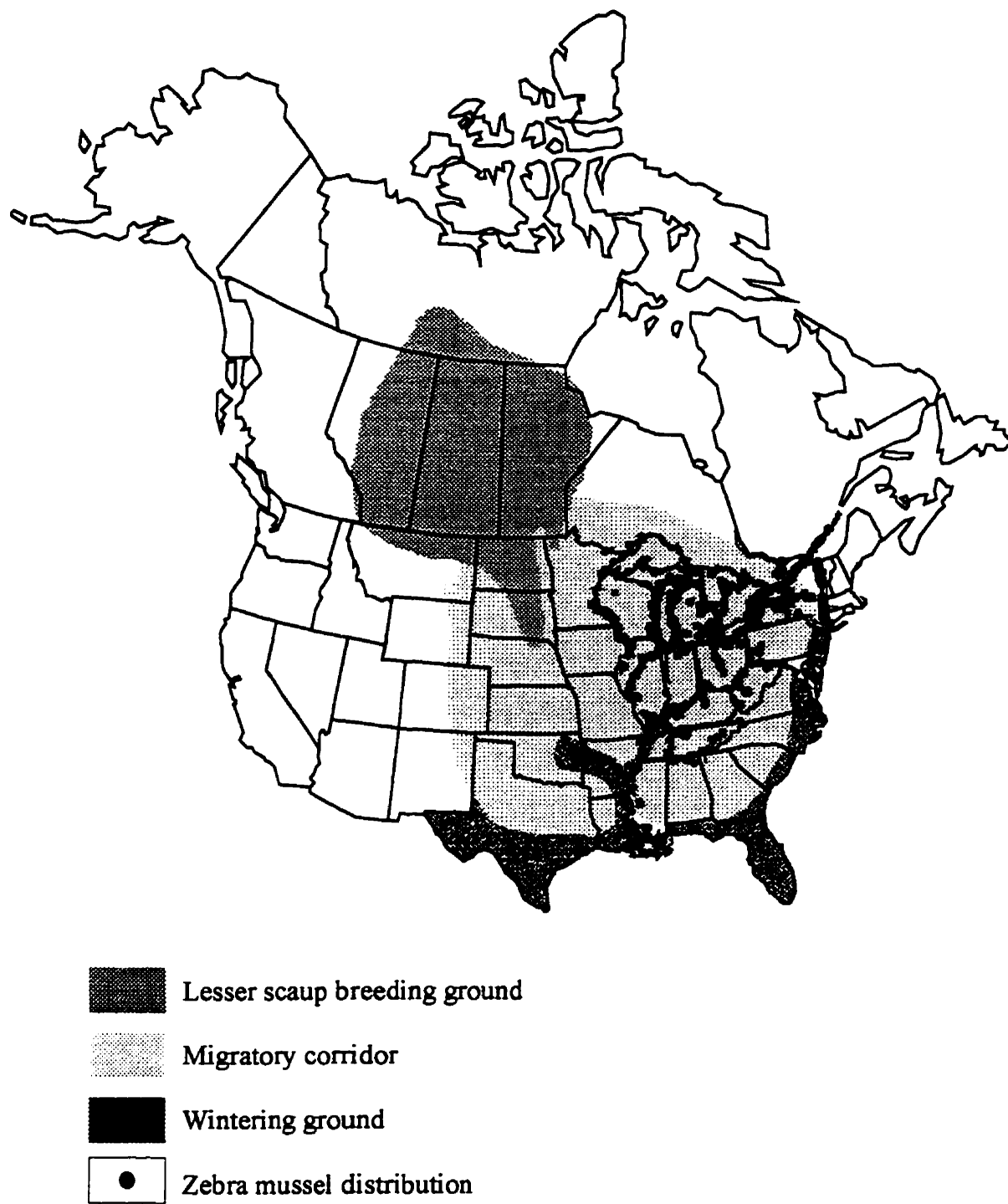


Figure 2.1. Overlap in zebra mussel and lesser scaup distributions.

been documented (Ricciardi *et al.*, 1995; MacIsaac, 1996). However, few studies have assessed the impacts of zebra mussels as a potential food source for different predators.

Dreissena are a main food source for several benthivorous fish, turtles and for waterfowl (Stanczykowska *et al.*, 1990; Prejs *et al.*, 1990; Hamilton, 1992; French III and Bur, 1993; Serrouya *et al.*, 1995). Fish with pharyngeal teeth such as the European roach (*Rutilus rutilus*) and the freshwater drum (*Aplodinotus grunniens*) are able to crush zebra mussel shells. In Europe, many diving ducks such as the tufted duck (*A. fuligula*), pochard (*Aythya ferina*), common goldeneye (*Bucephala clangula*), coot (*Fulica atra*), and the greater and lesser scaup (*A. marila* and *A. affinis*) feed intensively on mussels (Géroudet, 1966; Schmidt, 1975; Stanczykowska 1977, Petroli, 1981; Suter, 1982; Stanczykowska *et al.*, 1990). In North America, the feeding habits of some diving duck populations have changed radically (Hamilton, 1992; Custer, 1993) and migratory patterns have been altered in response to the proliferation of zebra mussels. For example, the lesser scaup population in Point Pelee National Park increased dramatically from a maximum of 150 ducks observed prior 1988 to 13 500 in 1989 (one day counts; Wormington and Leach, 1992) and the birds remained in the Park area much longer than they did prior to the arrival of zebra mussels (Hamilton *et al.*, 1994). Furthermore, the colonization of *Dreissena* in the wintering ranges of several molluscivorous duck species (Fig. 2.1) could permit them to feed on zebra mussels most of the year.

Previous studies demonstrated that zebra mussels rapidly bioaccumulate some organic contaminants in concentrations 30 000 times superior to those found in the surrounding waters (Snyder, 1990). Thus, they may alter the trophic structure and disrupt the existing food web dynamics in the Great Lakes basin. Given the known high filtration rates and high lipid contents of *Dreissena* (Dermott *et al.*, 1990), food chain transfer of contaminants could constitute a significant risk to predatory duck species that depend on *D. polymorpha* for their nourishment (Scholten *et al.*, 1989; de Kock and Bowmer, 1993).

2.2 Birds as sentinel species-The lesser scaup model

The marked decrease of wild bird populations due to epidemics or reproductive failure may be caused by xenobiotics. One of the first bird species to be studied was the peregrine falcon (*Falco peregrinus*). The decrease in the peregrine falcon abundance throughout most of the Holarctic resulting from eggshell thinning was positively related to persistent organochlorine pesticides (Hickey, 1969; Cade *et al.*, 1988). Mortality of rare species such as the burrowing owl (*Athene cunicularia*) was caused by heavy use of carbofuran in the Canadian prairies (Fox *et al.*, 1989). Starlings nesting in artificial nest boxes were affected by insecticide spraying (Grue *et al.*, 1982; Trust *et al.*, 1994). Reproductive failure of terns (*Sterna hirundo*) and herring gulls (*Larus argentatus*) on the Great

Lakes showed positive correlation with dioxin equivalents (Gilbertson, 1975; Hoffman *et al.*, 1987).

For waterfowl, the mallard (*Anas platyrhynchos*) is the most used duck species for the assessment of ecotoxicological impacts of heavy metals and organochlorines (Trust *et al.*, 1990; Mineau, 1991). A close relative of lesser scaup, the tufted duck, feeds extensively on zebra mussels in Europe and has been used as a sentinel species for assessing contaminant transfer and possible reproductive impacts of *Dreissena* (de Kock and Bowner, 1993).

The lesser scaup breeds most abundantly in wetlands of boreal forest in northern Saskatchewan, Manitoba and Ontario (Terres, 1987). However, the species seems to have enlarged its breeding ground toward the east with several sightings in northern Quebec (Gauthier and Aubry, 1995). It reproduces generally in lakes and ponds less than 2 ha in area and at least 3 m deep. Pairs are not territorial and contrary to males, females tend to come back to the same site every year. The female usually lays 8 to 10 eggs that it incubates for a average of 28 days. The young are precocious and fledge at 45-54 days (Gauthier and Aubry, 1995).

The lesser scaup is a expert diver. They project themselves underwater with their feet, with their wings tightly folded. Scaup feed mostly at depths of 1-2 m, but similar to the greater scaup, may also dive to 4-6 m deep. Before the arrival of zebra mussels, the diet of lesser scaup at their wintering areas along the eastern coast of the U.S. consisted of both aquatic vegetation (50%) and animal matter (50%) (Cronan, 1957). During migration and at breeding sites in

Manitoba, the main foods eaten were invertebrates (82%), mainly of Gammarus, Gastropoda, Tenebrionidae and Corixidae spp. (Rogers and Korschgen, 1966).

Zebra mussels have invaded most sites along the migration route and some wintering areas of lesser scaup populations on the east and south coast of the U.S. (Fig. 2.1) (Rand, 1971; New York Sea Grant, 1996). The availability of *Dreissena* has drastically changed the food habits of lesser scaup (Mazak, 1995; J. Barclay, pers. comm.). On some parts of Lake Erie, many scaups fed exclusively on zebra mussels (Hamilton *et al.*, 1994; Mazak, 1995). For these reasons, I chose the lesser scaup as a model species to assess the impact of a diet of zebra mussels on waterfowl health.

2.3 Contaminants under study

Dreissena polymorpha accumulate high levels of heavy metals (Kraak *et al.*, 1993) polycyclic aromatic hydrocarbons (Marvin *et al.*, 1994) and polychlorinated biophenyls (Brieger and Hunter, 1993; Doherty *et al.*, 1993). We focussed our attention mainly on the polychlorinated biphenyls (PCBs) and chlorinated hydrocarbon pesticides since they are the dominant contaminants of food webs in the Great Lakes-St. Lawrence River basin.

Cadmium, a non-essential metal, has the tendency to bioaccumulate in living organisms. Cadmium is widely distributed in industrialized aquatic systems (Coombs, 1979).

2.3.1 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls are a group of man-made aromatic compounds characterized by two benzene rings in which some of the hydrogen atoms have been substituted by chlorine atoms. Much concern has been expressed for their relatively high environmental persistence and toxicity.

PCBs are manufactured by chlorination of the biphenyl in the presence of a suitable catalyst. In their general formula, $C_{12}H_{10-n}Cl_n$, n is the number of chlorine atoms which can range from 1 to 10. A total of 209 different PCB components are possible, but only about 100 are commonly present in commercial mixtures (NRCC, 1981).

PCB mixtures are usually composed of several isomers with different amounts of chlorine and are chemically very stable under normal conditions. They have very low electrical conductivity, relatively high thermal conductivity and extremely high resistance against thermal breakdown, leading to their use as cooling liquids in transformers, capacitors, heat transfer systems, hydraulic equipments and vacuum pumps. They also have a very low solubility in water but dissolve readily in most organic solvents.

The most toxic PCBs have a coplanar structure resembling those of dioxins and furans and they also bind to the same cytosolic *Ah* receptor. The *Ah* receptor is part of a receptor-mediated system in which the inducing chemical binds first to this receptor. The receptor-inducer complex then binds to regulatory elements in the DNA, stimulating transcription of mRNA.

Aquatic systems are the ultimate sink for PCBs. Due to their highly lipophilic nature, these compounds tend to be strongly adsorbed into most sediments and suspended particles in aquatic systems. Zebra mussels may absorb these particles through filter feeding and store them in their lipid reserves. Several workers have speculated that *Dreissena* could be an important vector in the transfer of contaminants to higher trophic levels (Landrum et al., 1990; Kraak et al., 1991; Kreis et al., 1991).

Animal studies indicate that long-term exposure to small doses of PCBs may induce tumors and cause adverse effects on reproduction and on several organs and systems (WHO, 1987). PCBs are potent inducers of microsomal enzymes. They enhance the metabolism of steroids such as estradiol and

androsterone which can lower fertility and reduce the hatchability of eggs in birds. In fact, Sholten and co-workers (1989) observed a reproductive failure in tufted ducks fed with naturally contaminated zebra mussels. Biomagnification values for DDE, total PCBs and Hexachlorobenzene (HCB) were 8,4, and 3 times higher in the ducks compared to zebra mussels, respectively. PCBs cause atrophy of lymphoid tissue in chickens and suppress a variety of humoral and cell-mediated immune responses (Lu, 1991).

2.3.2 Chlorinated Hydrocarbons

Chlorinated hydrocarbons used in pesticides, include DDT, DDD, DDE, aldrin, dieldrin chlordane, mirex and Nonachlor. Most of these compounds are neurotoxicants with low vapor pressures and most are highly persistent. They are not readily absorbed across the skin barrier, but they pass across the gill integument in fish and are easily absorbed by the digestive tract of birds and mammals (Peterle, 1991). DDT is a well known toxicant due to its high chemical stability and high lipophilicity (O'Brien, 1967). The half life of DDT has been estimated as 10 to 20 years (USEPA, 1975). DDT metabolizes to DDE, which is also able to produce toxic effects in most species. DDE may be converted by epoxidation to more toxic substances which may be mutagenic and carcinogenic (Eisler and Jacknow, 1985).

DDT and its metabolites are the most studied chlorinated hydrocarbon compounds. Several studies demonstrated lethal and chronic effects of DDT on

avian populations. Widespread reduction in American robin (*Turdus migratorius*) populations was reported in urban areas being treated with DDT for Dutch elm disease (Bernard, 1963). Eggshell thinning has now been demonstrated in at least 54 species of birds from 10 orders, especially birds of prey (Ratcliffe, 1967; Stickel, 1975). Furthermore, DDT enhances hepatic microsomal activity, alters enzyme production, and decreases both calcium transport and estradiol levels in the blood (Bitman *et al.*, 1969; Peakall, 1970; Dieter, 1974; Richie and Peterle, 1979).

2.3.3 Heavy metals—Cadmium

Cadmium is a common environmental pollutant with a half-life (20-30 years) and a vapor pressure higher than those of other metals. Cadmium is present as a soluble substance in water and food and in particulate form from the emissions of welding, smelting, electroplating, and automobile exhausts. Cigarette smoke, pigments, pesticides and metal alloys are also significant sources of cadmium (Peterle, 1991). Cadmium is of concern because of its high toxicity and bioavailability as a non essential metal for the organism. Some species such as bivalve mollusks are good accumulators of heavy metals (Tessier *et al.*, 1994). The route of Cd uptake in mussels is through filtering. The metal crosses the cell membrane by facilitated diffusion via calcium pumps (Rainbow and Dalliger, 1993).

The primary toxic effects of this metal are those associated with renal, hepatic and testicular damage and bone decalcification. Cd is a potent immunotoxicant in that it increases susceptibility to bacterial and viral infections, inhibits humoral immunity, and modulates lymphocyte proliferation (Koller, 1979; Koller *et al.*, 1979).

2.4 Biomarkers

2.4.1 The rationale behind using biomarkers

Chemical monitoring has been in use to assess environmental contamination. Although this kind of monitoring may identify potential contaminant-related adverse effects on biota, it does not detect or describe those effects. As Reynoldson *et al.* (1989) stipulated, "there has been a developing awareness that chemical objectives alone are insufficient as indicators of overall health of the ecosystem, and that ultimately, the biological integrity of the ecosystem is the prime concern". Hence, there is a growing need to develop methods and techniques to determine the effects of the ambient levels of contaminants on wildlife populations (Fox, 1993). Detecting and evaluating biological changes are essential to determine the environmental significance of xenobiotic exposure. This is how biomarkers, as a measure of contaminant-associated effects, evolved. A biomarker is a xenobiotically-induced variation in cellular or biochemical components of processes, structures, or functions that is

measurable in a biological system or sample (NRC, 1987). However, in recent years, the term biomarker was also used at higher levels of biological organization, from the biochemical to populations and even ecosystems. Changes at the biochemical level offer distinct advantages as biomarkers for two main reasons. First, alterations at this level are usually the first detectable, quantifiable responses to environmental contaminant-induced stress. Second, biochemical alterations can serve as early indicators of both exposure and effect of xenobiotics which may later have impacts on higher levels of organization (e.g. populations and communities) (Stegeman *et al.*, 1992).

2.4.2 The need to validate “laboratory” biomarkers in environmentally relevant conditions.

Natural populations are exposed to a mixture of chemicals rather than to single xenobiotics. Organisms are subjected to many stresses not normally encountered in laboratory conditions (e.g. heat and cold stress, nutritional and energy deficiency, migration stress and prey-predator stress). Furthermore, animals within a same population may respond differently to the same chemical exposure depending on individual idiosyncrasies, genetic variability, varying states of nutrition, and varying micro-environments (Depledge *et al.*, 1993). These and other variables may greatly confound extrapolation of conclusions gleaned in the laboratory to field situations.

The monitoring of trace pollution in freshwater systems is of major interest. Aquatic bryophytes, fish and various bivalve mollusks such as the

Asiatic clam (*Corbicula fluminea*) and the zebra mussel were shown to be successful bioindicators of pollution (Leglise and Crochard, 1987). Aquatic birds also have been used as bioindicator species. As a top predator at the end of the trophic chain, herring gulls have proven to reflect the contaminant burden of the Great Lakes system (Fox *et al.*, 1988).

Once the choice of bioindicator species is made, one must decide which potentially useful tools to use to detect either exposure to, or effects of, the chemicals under study. In order to objectively choose useful biomarkers, several criteria should be considered. Questions about the relative sensitivity, biological and chemical specificity, clarity of interpretation, time to manifestation of endpoint, permanence of response, applicability to field conditions and methodological considerations should be clearly answered before any attempt is made to use a biomarker (Mayer *et al.* 1992).

2.4.3 Porphyrin profile

Heme biosynthesis is an essential process in all cells. It meets the needs for synthesis of peroxidases and hemoproteins, such as hemoglobin and myoglobin and also for mitochondrial cytochrome synthesis which is an essential prerequisite for eukaryotic life (Huggett *et al.*, 1992; Day *et al.*, 1981). Reticulocytes and hepatocytes are the two major sites for heme synthesis in vertebrates (Marks *et al.*, 1982). When the porphyrin biosynthetic pathway is disturbed by exogenous agents such as organochlorines, the porphyrin profile is

altered. Abnormal proportions of porphyrin intermediates in liver and urine can interfere with the normal heme biosynthesis pathway. The multiplicity of symptoms of porphyria can range from severe photosensitivity and photomutilation through anemia to abdominal pain and paralysis (Day *et al.*, 1981).

Heme synthesis involves a sequence of condensations followed by a series of oxidations. The first step in porphyrin and heme biosynthesis involves the condensation of succinyl-CoA and glycine to form δ -aminolevulinic acid (ALA). This reaction is catalysed by ALA synthetase which is the regulatory reaction (rate-limiting) enzyme of the biosynthetic pathway. With the help of ALA dehydrase, two molecules of ALA condense to form porphobilinogen (PBG). Four molecules of PBG then condense to form uroporphyrinogen III (uro). The enzyme, uroporphyrinogen decarboxylase, catalyses the decarboxylation of Uro (via hepta-, hexa, and penta intermediates) into coproporphyrinogen (copro). Protoporphyrinogen (proto) is formed by oxidative decarboxylation of copro then the reaction is catalyzed by enzymatic oxidation of protoporphyrinogen to yield protoporphyrin. Finally, iron chelation to the protoporphyrin ring by the enzyme ferrochelatase gives heme. These reactions are catalyzed by the enzymes present in the microsomal membranes and the cytosol fraction (Maines, 1984)

Exposure to organic xenobiotics are known to alter the porphyrin profile in birds. Vos and Koeman (1970) were the first to report that the oral administration of a PCB to chickens can cause hepatic porphyria which is characterized by urinary excretion and accumulation of uroporphyrin in the liver. Since then,

several organochlorine preparations have been shown to cause porphyria in various bird species (Carpenter *et al.*, 1984; Miranda *et al.*, 1987 & 1992; Akins *et al.*, 1993). The mechanisms yielding porphyria are well understood (Sano *et al.*, 1985). The porphyrin profile has been used extensively as a biomarker to evaluate the biochemical impacts of organochlorine mixtures on wild bird populations (Gilbertson and Fox, 1977; Fox *et al.*, 1988; Rattner, 1989). Several PCBs have been shown to inhibit uroporphyrinogen decarboxylase, leading to a depletion of heme and subsequently to an increase of ALA synthetase synthesis through feedback responses (Sano *et al.*, 1985). These disorders may contribute to poor hatching success in birds. Gilbertson and Fox (1977) postulated that porphyria may have contributed to pre-pipping death in herring gull embryos.

The most potent porphyrin-inducing polychlorobiphenyls are those which possess a coplanar structure (dioxin-like), and chlorine atoms at *para* and *meta* positions. In a study done by Sano and co-workers (1985), 3,3',4,4'-tetrachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl induced 200 and 262 times more porphyrin formation in chick embryo liver cells than occurred in controls.

2.4.4 Ethoxyresorufin-o-deethylase (EROD)

The elimination of fat-soluble pollutants like PCBs depends upon their conversion to water-soluble products that can be readily excreted. The

metabolism of these fat-soluble organochlorine compounds depends upon the action of the mono-oxygenase enzymes of different hepatic cytochrome families (Walker *et al.*, 1987). Induction of these enzymes is a parameter that has been proposed as an early-warning system for identifying environmental contamination threats in wild bird species (Boersma *et al.*, 1986; Peakall *et al.*, 1986; Hoffman *et al.*, 1986, 1987; Rattner *et al.*, 1989).

Cytochrome P450 is a family of enzymes that transform the structure of organic chemicals including halogenated hydrocarbons. Exposure of mammals and birds to different exogenous compounds results in increased levels of hepatic and extrahepatic Phase I and Phase II (conjugating) drug-metabolizing enzymes. Phase I includes several cytochrome P-450-dependent microsomal mixed-function oxidase enzymes catalysing reactions such as dealkylation and oxidation. These enzymes biotransform both endogenous and exogenous (foreign) compounds. Endogenous reactions involve the synthesis and degradation of steroids, prostaglandins, fatty acids, and other biological molecules. P450 also transforms foreign compounds such as halogenated aromatic hydrocarbons which may facilitate their excretion. Certain enzymes such as EROD and aryl hydrocarbon hydroxylase (AHH), from the family P4501A, are highly specific in their response to certain PCBs. EROD is responsible for the dealkylation of ethoxyresorufin to resorufin via P4501A-dependent monooxygenase system (Boersma and Ellenton, 1986).

The metabolism of commercial PCB mixtures is enhanced by phenobarbitone (PB)- and/or 3-methylcholanthrene (MC)-induced microsomal

enzymes (Safe, 1984). Some dioxin-like PCBs (coplanar congeners) exhibit a MC-induction pattern and induce hepatic microsomal EROD and AHH (Parkinson *et al.*, 1983). There is now strong evidence that measuring the induction of P4501A proteins (EROD) is a valuable approach for sensitive and early detection of chemical exposure and effect (see review by Huggett *et al.*, 1992).

2.4.5 Vitamin A concentrations

The different molecular forms of vitamin A are referred to as retinoids. Some retinoids are biologically active while others serve as stores that can be converted to biologically active forms. Retinyl palmitate (storage form of vitamin A) is quantitatively the most important retinoid in vertebrates and relatively high concentrations are usually stored in the liver (Olson, 1981). Retinyl palmitate is mobilized from the liver in response to the physiological demand. Retinyl palmitate is converted to retinol (active form of vitamin A) which, in turn, is bound to retinol binding protein (RBP), released from the liver and transported in the blood to peripheral tissues.

Retinyl palmitate and retinol have been identified as the two main retinoids affected by dietary deficiency and/or exposure to organic pollutants. In fact, the effects of nutritional vitamin A anomalies are strikingly similar to those elicited by the exposure to dioxin-like compounds. In birds and mammals, vitamin A deficiency has been shown to affect vision, bone growth, differentiation and

proliferation of epithelial tissues, and leads to teratogenic effects (Thompson, 1976; Roberts and Sporn, 1984).

Poultry fed vitamin A-deficient diets showed behavioural depression, poor body conditions, nasal and ocular discharge, squamous metaplasia of the mucosal epithelium, decreased egg production and lowered hatchability of fertile eggs once liver stores of retinyl palmitate became depleted (Sommer, 1990). Vitamin A has been called the "anti-infection vitamin" because deficiencies increase susceptibility to infectious diseases. Over-wintering mallards (*Anas platyrhynchos*) in Saskatchewan during 1990-91 died of bacterial infections when forced to feed on a vitamin A-deficient diet of cereal grains. Necropsies revealed squamous metaplasia of the submucosal glands of the esophagus which might have been the portal of entry for *Staphylococcus aureus* bacterial infections (Wobeser and Kost, 1992). Fox (1993) suggested that marked depletion of vitamin A in gulls nesting in the western part of Lake Erie could increase their susceptibility to infectious diseases and affect embryo viability and development.

It is well known that polychlorinated biphenyls, polyhalogenated dibenzo-*p*-dioxins and dibenzofurans elicit an array of effects upon the dynamics and metabolism of retinoids (Thunberg *et al.*, 1979; Spear *et al.*, 1986, 1989, 1990; Mercier *et al.*, 1990). Spear and co-workers (1986) reported that a relatively low dose of the dioxin-like PCB, 3,3',4,4'-tetrachlorobiphenyl, decreased the concentration of retinol in the liver of doves in laboratory conditions. Furthermore, herring gull livers from contaminated colonies in the Great Lakes

showed lower retinyl palmitate and retinol concentrations when compared to colonies living in low-pollution areas (Spear *et al.*, 1986; Government of Canada, 1991). The accelerated retinoic acid catabolism (degradative metabolism) through cytochrome P450 hydroxylation and UDP-glucuronyltransferase conjugation reactions may be responsible for the lower vitamin A concentration in exposed birds (Spear *et al.*, 1990). Moreover, alterations in the activity of retinyl ester hydrolase, the enzyme responsible for the hydrolysis of the storage form of vitamin A (retinyl palmitate) into free retinol, may also contribute to the depletion of vitamin A (Mercier *et al.*, 1990).

2.4.6 Histopathological hepatic biomarker

Histopathological biomarkers are lesions that signal effects resulting from prior or ongoing exposure to one or more toxic agent(s) (Braunbeck *et al.*, 1992). They are higher level responses, reflecting prior alteration in physiological and/or biochemical function. Most of these biomarkers are lesions demonstrated in laboratory toxicant exposures. Field investigations of histopathological lesions (biomarkers) are rare and have yet to demonstrate their relevancy and usefulness as potential biomarkers (Hinton and Laurén, 1990).

Exposure to xenobiotics may lead to toxicant-induced necrosis (death of the cells) or may result in cellular injury. When the concentration of a toxicant is sufficient to result only in cellular injury but not death, sublethal (adaptive) changes may be observed in affected cells (Trump *et al.*, 1980). Sublethal

changes include cellular swelling (hydropic degeneration), accumulation of cytoplasmic inclusions, and changes in cell and nuclear volume. Lipid accumulation in the cytoplasm of affected cells is a common indicator of subacute and chronic toxicities (Dixon, 1982). Glycogen depletion often affects hepatocyte staining since this complex carbohydrate does not stain with conventional methods and normally occupies large portions of the cytoplasm (Hinton *et al.*, 1992).

The liver is the most studied organ for lesions (Myers *et al.*, 1990; Vogelbein *et al.*, 1990). The main reasons are that: 1) absorbed nutrients and toxicants from the gastrointestinal duct are stored in hepatocytes (Walton and Cowey, 1982), 2) the liver is the major site of the cytochrome P450-mediated system which inactivates some xenobiotics, while activating others to their toxic forms (Stegeman *et al.*, 1979), and 3) the yolk protein, vitellogenin, which is synthesized entirely within the liver (Vaillant *et al.*, 1988), has first to be initiated by the binding of estradiol (or estradiol-like contaminants) to hepatic receptors.

Presented below are some of the most common hepatic histopathological lesions used as biomarkers because they result from exposure to xenobiotics.

Coagulative hepatocellular necrosis, associated with sudden cessation of blood flow to an organ, has positively been associated with exposure to anthropogenic environmental toxicants (Meyers and Hendicks, 1985; Pitot, 1988). Following necrosis, surviving cells undergo hyperplasia which allow the regeneration of needed replacement hepatocytes. The regenerating cells are small and basophilic, forming small islands of irregular shape. *Hyperplasia* is

indicative of extensive regeneration prior necrosis which may be caused by toxicant exposure (Zuchelkowski *et al.*, 1986).

Bile ductal hyperplasia refers to numerous and contiguous bile ductules with abundant branching and coiling. This lesion is of chronic duration and has been consistently found in wild fish from chemically contaminated sites (Hayes *et al.*, 1990). In western Lake Ontario, proliferative biliary disease of white suckers (*Catostomus commersoni*) were associated with bile duct neoplasms in polluted harbors (Hayes *et al.*, 1990).

Hepatocellular hypertrophy is a type of *hepatocytomegaly* characterized by organelle hyperplasia within the cytoplasm of hepatocytes, with enlarged cellular diameter. This condition may lead to a net gain in the tissue mass of an organ. Channel catfish (*Ictalurus punctatus*) exposed to Aroclor 1254 showed a hypertrophy of the endoplasmic reticulum (Klaunig *et al.*, 1979). Another type of hepatocytomegaly is the presence of cells with a clear cytoplasm, large vacuoles and small compact nuclei. This lesion is referred to as hepatocellular vacuolation and was observed in fish caught in polluted harbors (Gardner *et al.*, 1989; Stehr, 1990).

2.4.7 Non-Specific Immunological Assays

Immunotoxicology has become of major interest due to the importance of the immune response in maintaining the integrity of the organism. Immunity is

the organism's first line of defense against external stressors and is known to be affected by several pollutants (heavy metals, PCBs, PAH) (Vos, 1977).

Studies have demonstrated that exposure to toxic chemicals interfere with early events in the capacity of immunocompetent cells to proliferate rapidly following activation with antigens, mitogens or hormones (see review by Weeks *et al.*, 1992).

Leukocytes mediate immune functions via both non-specific and specific immune responses. Nonspecific immunity is mediated by mononuclear phagocytes (blood monocytes, tissue macrophages) and granulocytes, which recognize foreign material. Specific immunity is mediated by several effector leukocytes such as the T- and B-lymphocytes, which are directed against specific antigens (Abbas *et al.*, 1994).

Non-specific immunity comprises two types of responses: the phagocytosis/respiratory burst which is the ingestion and destruction of foreign agents and the inflammation which accompanies the phagocytic infiltration process at the site of injury or infection. Polymorphonuclear leukocytes (neutrophils and heterophils in birds) and mononuclear phagocytes (macrophages) are the main cell types responsible for non-specific immune responses (Weeks *et al.*, 1992). Heterophils are numerous short-lived cells that are specialized in the ingestion (phagocytosis) and destruction (respiratory burst) of microorganisms in the blood or in tissues of birds following infiltration of an infection site by chemotactic stimuli (Tizard, 1992).

Some non-specific assays such as the determination of leukocyte number, blood differential counts, phagocytic activity, and lysozyme and peroxidase levels have been used mainly in the wild for assessing fish health (Warinner *et al.*, 1988). Lysozyme and oxidative burst are important in the destruction of invasive agents and in some cases can serve as an early biomarker indicating deterioration of some protective mechanisms (Peters *et al.*, 1990). Phagocytosis is well conserved throughout phylogeny and is an important part of the immune surveillance in invertebrates as well as vertebrates. Therefore, assays of these immune functions have potential as bioindicators of toxic effects in animals (Weeks *et al.*, 1992).

Fish exposed to polynuclear aromatic hydrocarbons (PAH) in a polluted river showed immunosuppression of macrophage activity (Weeks *et al.*, 1987). Earthworms exposed to PCB for up to 122 h showed significant reductions in phagocytic activity of coelomocytes (Goven *et al.*, 1988). Exposure to dieldrin, mercury, lead and cadmium in mice and rats were also shown to affect phagocytosis (Loose *et al.*, 1978; Krzystyniak *et al.*, 1985; Brunet *et al.*, 1993) and respiratory burst activity (Krzystyniak *et al.*, 1987; Contrino *et al.*, 1992).

Increased susceptibility to viral, bacterial and parasitic infections in animals exposed to immunotoxic chemicals can usually be related to the immunosuppressive potential of the chemical (Wong *et al.*, 1992). In chickens and mice, DDT and PCBs increased susceptibility to parasitic and viral infections (Koller, 1977; Koller, 1979; Faith *et al.*, 1980). Organochlorine pesticides (DDT and dieldrin) have been shown to affect resistance to viral hepatitis in ducks

(Friend and Trianer, 1974). Waterfowl are very vulnerable to immunotoxicant enhancement of disease because they frequent waters and feed in areas in which xenobiotics tend to accumulate, and they are highly gregarious, facilitating the spread of pathogens (Rocke *et al.*, 1984).

2.4.8 Metallothionein– Biomarker of Metal-induced Stress

Metallothionein (MT) is a metal binding protein which is characterized by a low molecular weight, a large proportion of cysteine residues, and a lack of aromatic amino acids (Vallee, 1991). Other intrinsic properties of MT include resistance to thermocoagulation and acid precipitation, properties that are often exploited in the separation of these polypeptides from high molecular weight proteins (Kagi and Schaffer, 1988). This metal binding protein is involved in the sequestration and the metabolism of heavy metals via cysteine thiolate linkages. The order of binding affinity between the cysteines and various metals ($Zn < Cd < Cu < Hg$) reflects the relative strength of the metal-thiolate bond. The strongest metal-inducer of MT synthesis is cadmium (Vazak, 1991) and MT is the only specific Cd-binding protein (Kagi and Schaffer, 1988). Essential metals (Zn^{2+} , Cu^{+}) and toxic heavy metals (Cd^{2+} , Hg^{2+}) increase the synthesis rate of MT messenger-RNA via transcriptional activation of metal-responsive factors located in the upstream region of MT (Labbé *et al.*, 1993).

Metallothioneins occur throughout the animal kingdom, as well as in plants and eukaryotic microorganisms. The organs in which MTs are concentrated in

aquatic animals are the liver, kidneys, gills and intestines (Roesijadi, 1992). MTs are involved in metal metabolism, such as metal detoxication, regulation of zinc and copper, and transfer of metals to metalloproteins (Brouwer *et al.*, 1989). MTs also play a role in the response to stresses such as cold, heat, exercise, and are an effective free-radical scavenger (Oh *et al.*, 1978; Thornalley and Vasak, 1985).

Induction of metallothioneins is one of the most studied biochemical responses of animal cells exposed to heavy metals (Engel and Brouwer, 1989; Vallee, 1991). Many studies have reported that elevated metal concentrations in the aquatic environment are correlated with the presence or concentrations of metals bound to MT in invertebrates (Roesijadi, 1981). A wide variety of invertebrates synthesize MT upon exposure to cadmium (Engel and Brouwer, 1989). In the freshwater bivalve *Anodonta grandis*, metallothionein levels were significantly correlated with tissue Cd concentrations. Free Cd²⁺ concentration at the sediment-water interface was the environmental factor to which metallothioneins responded (Couillard *et al.*, 1993). MTs in the gills of mollusks are induced by cadmium, copper, and mercury and sequester a significant portion of metals accumulated by this organ (Viarengo *et al.*, 1980; Roesijadi, 1982:). In a limpet (*Patella vulgata*) collected from a site contaminated by cadmium, the amount of cadmium bound to MT in the soft tissues was directly correlated to the tissue cadmium concentration and the size of individuals (Noël-Lambot *et al.*, 1980).

For the studies mentioned above, induction of MTs *in situ* most likely reflects mobilization of a compensatory response associated with metal sequestration and detoxification. Thus, proposals for the use of MTs as biochemical indicators of metal pollution in aquatic environments merit continued consideration (McCarthy and Shugart, 1990). The main attractions of using such cellular indicators are their sensitivity, specificity, and potential usefulness as early warning systems of adverse effects (Roesijadi, 1992).

CHAPTER 3

ARTIFICIAL INCUBATION AND BROODING

OF LESSER SCAUP, *AYTHYA AFFINIS*

3.1. Abstract

Recent decreases in waterfowl populations have led us to improve incubation and brooding methods to permit the study of diving ducks in captivity. Lesser scaup was chosen as a wild species model to assess the potential health impacts of the consumption of a contaminated new food source, the zebra mussel (*Dreissena polymorpha*). An egg transportation device, artificial incubation methods, hatching, and captive rearing techniques for lesser scaup are described. High hatching success (88.6%) was achieved using Roll-X incubators with daily egg cooling and water misting. Appropriate feeding, bathing, and hygiene precautions yielded low (3%) post-hatching mortality.

3.2. Introduction

There has been a decrease in populations of several waterfowl species over the past 20 years (Dickson, 1989; Wahle and Barclay, 1993; Grand, 1995). Exact causes for decline are not yet known but it is thought that a combination of factors is involved. The loss of wetlands, pollution burdens, predators and hunting pressure are potential causes (Dickson, 1989).

Lesser scaup (*Aythya affinis*) populations in North America have steadily declined since the late 1950's (Wahle and Barclay, 1990, 1993). More recently, the zebra mussel (*Dreissena polymorpha*) invasion of the Great Lakes has had a serious impact on lesser scaup, altering their migratory routes and food habits (Hamilton et al., 1994; Mazak, 1995). A zebra mussel diet, which is characterized by low nutritive value and high contaminant level (Secor *et al.*, 1993), may partially explain the decrease of some lesser scaup populations in recent years. To test this hypothesis, lesser scaup was chosen as an animal model to assess possible impacts of the zebra mussel on diving duck health. Few wild species have been used as models in ecotoxicological food web studies (Fox, 1993).

Waterfowl eggs are known to be more difficult to artificially incubate than poultry eggs (Korthang, 1986). There are few suggestions in the literature (Ward and Batt, 1973; Dayton, 1974) of efficient and inexpensive ways to transport and incubate diving duck eggs taken from the wild. Furthermore, the different variables (humidity, temperature and length of incubation) important for

successful waterfowl egg incubation have not been adequately determined for many North American species, including the lesser scaup.

This paper describes a simple, inexpensive, and successful way to transport, incubate, and hatch lesser scaup eggs as well as brooding and rearing techniques. Hatching success and problems encountered during our incubation and brooding of young ducklings are also discussed.

3.3. Materials and Methods

Thirty-five eggs with embryo movements and well developed yolk sac blood vessels were collected from 6 different lesser scaup nests in the vicinity of Saskatoon, Saskatchewan on June 29, 1994. The eggs were carefully washed with lukewarm water.

Eggs were placed in a padded egg container in a Styrofoam box (70 cm x 35 cm x 46 cm). Two hot water bottles (~45°C) were placed at the bottom to provide warmth and the egg containers were strapped in the middle part of the box to permit air circulation. A wet cloth was spread over the hot water bottles to maintain high humidity. A temperature probe was placed inside the egg containers with the thermometer (Taylor, Canada) attached to the outside of the Styrofoam box. The temperature could be easily read from the outside without opening the box, preventing subsequent loss of heat and humidity. The water in the hot water bottles was replaced when the temperature dropped below 32°C.

The eggs were stored in the Styrofoam box for approximately 10 hours until arrival at the Avian Science and Conservation Centre, Macdonald Campus, McGill University, Ste. Anne de Bellevue, Québec.

Upon arrival, the eggs were transferred to a prefumigated Roll-X artificial incubator (Marsh Farms, Garden Grove, CA) equipped with an automatic turner. The incubators were fumigated using formaldehyde gas by mixing 0.4g of potassium permanganate (KMnO_4) with 0.8 ml of formalin (37.5%) per cubic foot of incubated volume. This fumigation procedure killed the bacterial organisms in the incubators. Eggs were placed in the incubator grid with the large end up. Incubator temperature was held constant at 37.5°C while the humidity was adjusted (~85%) to ensure adequate weight loss. Room temperature was maintained constant at 20°C. Eggs were turned automatically every hour.

The incubator was left open for a period of 10-15 minutes every afternoon and a gentle mist of warm distilled water was sprayed on the eggs.

All eggs were candled once a week to ensure their viability. The eggs were weighed every three days to the nearest 0.01g using an electronic balance and their weight loss was monitored using an egg weight-loss graph (Campbell and Flood, 1977; Heck and Konkel, 1983). The formula used to estimate the egg weight at laying (W) in grams was derived from Hoyt (1979):

$$W = L * T^2 * K_w$$

where L is the egg length (mm), T, the egg width (mm) and Kw, the observed weight coefficient. Since no Kw was specifically determined for the genus *Aythya*, the Kw of mallard, *Anas platyrhynchos* (0.000560) (Hoyt, 1979) was used.

To calculate the daily mean water loss (M_{H_2O}) the following equation was used:

$$M_{H_2O} = W * 0.15 / 28$$

where 28 represents the mean incubation period in days for lesser scaup (Hyde, 1974). The percentage of weight loss from laying to hatching was set at 15% (Ar and Rahn, 1980; Burnham, 1983; Heck and Konkel, 1983).

To estimate at which stage (in days) of incubation (Is) the egg was at, the following equation was used:

$$Is = (W - X) / W * 0.141 / 28$$

where W is the egg weight (g) at laying and X is the actual weight of the egg (g).

After pipping, the eggs were transferred to a hatcher. The hatcher is simply a Roll-X incubator with the sliding portion of the turning grid removed. The lower portion of the turning grid is covered with a piece of 1/4" hardware cloth, which in turn is covered with crinoline hatching fabric. Temperature in the hatcher was reduced by 0.5°C to 37°C and the bottom of the incubator flooded with distilled water to achieve the maximum humidity possible. The date and time of

transfer were recorded to ensure that eggs did not stay in the hatcher for more than 72 hours. Most chicks hatch within 48 hours of pipping (Ward and Batt, 1973). Eggs which had not hatched 48 hours after the initial pip were assisted out of the shell. The shell was gently flaked around the air chamber using small forceps. Great care was taken to ensure blood vessels were not cut. We followed Greenwell's (1974) protocol for helping ducklings out of the egg.

Ducklings remained in the hatcher until their down was completely dry. They were then transferred and reared in a 3m x 3m enclosures bedded with wood shavings in 2 separate rooms. This was to ensure that, if any diseases or infections appeared in one group, it would not affect or spread out to the second group located in the room. Heat lamps suspended over the ducklings provided an external heat source. Initially the temperature was kept at 35°C at ground level directly under the heat lamps. This was gradually reduced to 20°C over a two week period, at which point the ducklings did not require an external heat source. Food and water were available *ad libitum* in commercial poultry feeders and waterers. The feeders and waterers were cleaned daily with an anti-bacterial soap and any wet bedding was removed. Ducklings were fed a commercial duck-starter diet (19% crude protein) for two weeks followed by a duck-grower diet (17% crude protein) (Nutribec, # 4281 and #4282, Québec, Canada).

Twice a day, ducklings were allowed to bath in fresh water basins (2 m diameter, 40 cm depth) and were fed live mealworms (*Tenebrio molitor*) to stimulate natural feeding behavior.

Ducklings were transferred to external pens (8m x 7m x 2m) (Ecomuseum, Ste. Anne de Bellevue, Québec) at approximately three weeks of age. The pens were made of nylon netting with aluminium siding to exclude predators. A concrete V shaped water basin (8m x 3m) with a maximum depth of 40 cm filled the central portion of each pen while the remaining area was grass. Wooden shelters were also provided. Rubber carpets covered the shallow water section on each side of the basin to prevent the pododermatitis (bumble foot) often encountered in diving ducks in captivity. A water pump (Jacuzzi, model 7L-S) and a sand filter with dial valve (Jacuzzi, flow rate 20 US gpm/sq ft) were installed in the basins to ensure adequate water flow and to minimize fungal and bacterial development. The sand filter was washed daily. All pens were thoroughly cleaned 3 times/week with a brush and water.

3.4. Results and Discussion

3.4.1. Transport and transfer container

The average temperature in the Styrofoam box was $35^{\circ}\text{C} \pm 3^{\circ}\text{C}$. The hot water bottles were refilled only once during the 10 hour period that eggs stayed in the transfer container. This easily constructed and inexpensive portable device proved to be quite reliable and maintained a stable temperature for long periods of time. Furthermore, no specialized parts were required and all the materials used were readily available at any hardware store.

Air travel with the transfer container may be problematic. Most commercial airlines we contacted were reluctant to allow the container to be brought into the passenger compartment. However, travelling in the same compartment as the eggs is a must given the potential for damage to fragile objects in a standard luggage compartment. One must also ensure that all the appropriate permits and authorizations to be able to travel with "live animals" are obtained in advance. In Canada, these include provincial export and import permits mandated by The Wildlife Act and Regulations and Canadian Wildlife Service. An Animal Care Certificate is also required.

3.4.2 Incubation

Only well vascularized eggs with live embryos were collected. These traits are readily visible in embryos which have received 7 or more days of incubation (Anderson-Brown, 1979). The first week of embryological development is very critical and natural incubation should prevail during this period (Searles, 1974). Duck eggs that received one to two weeks of natural incubation showed higher hatchability with artificial incubation (Orr, 1978).

Daily cooling and spraying of the eggs mimics natural conditions where the female leaves the nest for short periods of time and returns with water on its plumage (brood patch), thereby increasing the nest's humidity which probably in turn increases the chance of successful hatching. Bauer (1974) reported that duck females from several species complete their preening over their nest as they returned from the water, possibly dropping moisture on the eggs. Some

breeders also cool and spray eggs but less frequently (every second or third day)(Ellis, 1974). It has also been reported that misting the eggs may help dissolve calcium in the shell, making it easier for the embryo to pip at hatching time (Reinhart, 1983).

3.4.3. Hatching

Seventy-two percent of the eggs hatched within 4 days of the predicted hatching date. Vocalisation during pipping and hatching of the first eggs probably stimulated the other ducklings to hatch earlier than expected. Synchrony in hatching is characteristic of several avian species (McFarland, 1985).

Hatching success was 88.6%. This percentage is considered high as many aviculturists are satisfied with any value $\geq 70\%$ (Anderson-Brown, 1979). The Delta Waterfowl Research Station obtains about 75 percent hatching success for fresh eggs, or eggs which get no natural incubation. In the case of maternal incubation for an initial 6-day period, Delta achieves a hatching success in the order of 90 percent (Ward and Batt, 1973). In the wild, hatching success rarely exceeds 50% (Rogers, 1964). Predation and adverse natural conditions are the main causes of the poor success (Rogers, 1964).

The high hatching success reached in this study is probably the result of strict hygiene (fumigation and disinfected incubator room) as well as close control and monitoring of variables affecting embryo development.

Of the three eggs that did not hatch, one partially pipped the shell without breaking the outer membrane. A necropsy revealed a partially retracted yolk sac

while the lungs were not fully developed, even though the embryo's head was inside the air chamber. The two other embryos never developed to maturity and their yolk sacs were partially ripped open. The vibration caused by the transportation of the eggs may explain this pathology.

Six chicks had to be assisted in hatching. The main cause for most chicks not to be able to hatch by themselves is the drying of the inner membrane which prevents the chick from turning within the shell (Anderson-Brown, 1979). The temperature inside the hatching incubator was set at 37.0°C which is similar to the 36.9°C Reinhart (1983) and Flieg (1974) suggested for most waterfowl. The humidity within the hatching incubator fluctuated from approximately 85% to 90% which may not have been high enough. Korthang (1986) suggested that humidity should be raised to 90-95% after pipping. Spraying distilled water (not directly on the eggs) inside the hatcher would raise the humidity level to near saturation. Ellis (1974) reported that after pipping, eggs should be sprinkled with warm water twice a day until the ducklings begin to hatch. He also stated that the hatching incubator should not be opened more than twice daily, in order to maintain high and uniform humidity levels. To help the chick out, we suggest spraying lactated Ringer's solution directly on the inner membrane after removing some shell fragments from the air cell. This will accelerate the resorption of membrane blood vessels (Greenwell, 1974).

Some waterfowl producers are convinced that if ducklings are not healthy enough or strong enough to hatch on their own, they will not reach maturity (Ellis, 1974). However, our study demonstrates that ducklings receiving assistance

during the pip-to-hatch stage develop into healthy chicks and subsequently adults.

3.4.4. Brooding

Only one duckling died post-hatching. The possible cause for death was inanition. Only wood shavings were found in its gizzard. For unknown reasons this duckling did not learn how to feed properly even after all birds were shown how to obtain water and food pellets. Lee (1974) reported that some duckling species such as the ruddy duck (Oxyura jamaicensis) are more susceptible to ingesting litter materials, leading to their death.

Soon after hatching, another duckling was not able to feed itself sufficiently. Moreover, it was not able to stand in the water with others and had a tendency to lose body heat rapidly. Probably this chick was still too young to bathe. Ducklings that hatch under natural conditions absorb enough oil from the feathers of the incubating adult to be waterproof and swim quickly after hatching (Bauer, 1974). We recommend waiting three to four days before bathing the ducklings. Bathing can start after the birds preen by themselves, using their own oil. This duckling received subcutaneous injections of lactated Ringer's solution for initial rehydration and then was tube fed with lactated Ringer's solution and given a diet supplement (Nutripet) mix. It gained enough weight after a week to be able to feed by itself and rejoined the rest of the ducklings.

The utilization of water basins for duck rearing and brooding (referred to as the "wet" brooding method) used in this study and previously described by

Bauer (1974) for diving ducks has proven to be very successful. Ducklings that are able to swim regularly have a better functioning oil gland and are more natural looking and fluffy. Furthermore, when transferred to the wild or outside pens, the birds raised with the "wet" method do not soak up water as do those ducklings which have not received regular bathing or any access to water for swimming (the "dry" method). As well, they seem to be less stressed and more functional than birds raised without early access to water basins.

We demonstrated that, by careful monitoring and using good hygienic precautions, high hatching success and low post hatching mortality may be obtained. These goals can be reached using inexpensive and readily available equipment. The techniques for lesser scaup incubation and rearing described here will hopefully enhance the use of this species as an ecotoxicological wild animal model.

3.5. References

- Anderson-Brown, A.F. 1979. The incubation book. Wheaton & Co. Ltd., London, U.K. Pp.188-227.
- Ar, A. and H. Rahn. 1978. Interdependence of gas conductance, incubation length, and weight of the avian egg. In: Piper, J., (Ed.). Respiratory function in birds, adult and embryonic. Springer-Verlag, Berlin, Germany. Pp. 227-236,

- Bauer, R.V. 1974. Natural incubation of ducks and Bantam chickens for hatching ducks., In: D.O. Hyde (Ed.). Raising wild ducks in captivity. E.P. Dutton & Co., New York,. Pp. 88-90
- Burnham,W. 1983. Artificial incubation of falcon eggs. J. Wildl. Manage. 47:158-168.
- Campbell, C and T. P. Flood. 1977. Incubation in raptor breeding. Hawk Chalk 16:38-50.
- Dayton, O.H. 1974. Raising wild ducks in captivity. E. P. Dutton & Co. New York, 167 pp.
- Dickson, K.M. 1989. Trends in sizes of breeding duck populations in western Canada 1955-1989. Progress notes no. 186, Canadian Wildlife Service. 9 pp.
- Ellis, J. 1974. Using an artificial incubator. In: D.O. Hyde (Ed.). Raising wild ducks in captivity. E.P. Dutton & Co., New York. Pp.96-98.
- Flieg, G.M. 1974. Incubators and methods. In: D.O. Hyde (Ed.). Raising wild ducks in captivity. E.P. Dutton & Co., New York. Pp. 103-104.
- Fox, G.A. 1993. What have biomarkers told us about the effects of contaminants on the health of fish-eating birds in the Great Lakes. The theory and a literature review. J. Great Lakes Res. 19: 722-736.
- Grand, J.B. 1995. Nesting success of ducks on the central Yukon Flats, Alaska. Can. J. Zool. 73: 260-265.
- Greenwell, G.A. 1974. On helping ducklings out of the egg. In: D.O. Hyde (Ed.). Raising wild ducks in captivity. E.P. Dutton & Co., New York. Pp 99-102.

- Hamilton, D.J., C.D. Ankney, and R.C. Bailey. 1994. Predation of zebra mussels by diving ducks: an exclosure study. *Ecology* 75: 521-531.
- Heck, W.R. and D. Konkel. 1983. Incubation and rearing. In: J. D. Weaver and T.J. Cade (Eds). Falcon Propagation, a manual of captive breeding. The Peregrine Fund, Inc., New York. Pp 34-71.
- Hoyt, D.F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. *The Auk* 96:73-77.
- Korthang, C.F.H.F. 1986. The incubation of duck eggs. In: Farrell, D.J. and P. Stapleton (Eds), Duck production and world practice. University of New England. Pp 168-177.
- Lee, F.B. 1974. Artificial brooding of ducklings. In: D.O. Hyde (Ed.). Raising wild ducks in captivity. E.P. Dutton & Co., New York. Pp. 129-136.
- McFarland, D. 1985. Animal behaviour: Psychobiology, ethology and evolution. Longman Scientific & Technical, Essex, U.K. 576 pp.
- Mazak, E.J. 1995. Organic contaminant profiles of lower Great Lakes' waterfowl in relation to diet, with particular reference to *Dreissena polymorpha*. Master's thesis. University of Windsor. 111 pp.
- Orr, H.L. 1978. Duck and goose raising. Ministry of Agriculture and Food of Ontario. Publication 532. 55 pp.
- Reinhart, B.S. 1983. Incubation. Ministry of Agriculture and Food of Ontario. Publication 234. 24 pp.
- Rogers, J.P. 1964. Effect of drought on reproduction of the lesser scaup. *J. Wildl. Manage.* 28: 213-222.

- Searles, G. 1974. Incubation by the female duck. In: D.O. Hyde (Ed.). Raising wild ducks in captivity. E.P. Dutton & Co., New York. Pp 82-84.
- Secor, C.L., E.L. Mills, J. Harshbarger, H.T. Kuntz, W.H. Gutenmann and D.J. Lisk. 1993. Bioaccumulation of toxicants, elements and nutrient composition, and soft tissue histology of zebra mussels (*Dreissena polymorpha*) from New York State Waters. *Chemosphere* 26:1559-1575.
- Wahle, A., and J.S. Barclay. 1990. Trends in scaup populations and food habits. 46th Annual Northeast Fish Wildl. Conf. Abstracts. Northeast Association of Fish and Wildlife Resource Agencies.
- Wahle, A., and J.S. Barclay. 1993. Changes in greater scaup foods in Connecticut. *Northeast Wildlife* 50:69-76.
- Ward, P., and B.D.J. Batt. 1973. Propagation of captive waterfowl. Delta Waterfowl Research Station and Wildlife Management Institute, Washington, D.C. 64 pp.

CONNECTIVE STATEMENT LINKING CHAPTERS 3 AND 4

Following the development of the successful incubation and rearing methods, I began feeding Lake Erie and St. Lawrence River zebra mussels to two separate groups of 10 three-week old lesser scaups. A third group was fed with a non-contaminated diet of duck chow. Throughout the experiment, blood sampling was done every three weeks. In the next chapter, phagocytosis and oxidative burst, two responses from the non-specific immunity, were assessed as potential biomarkers. Immunotoxicological assays are relatively recently introduced biomarkers of potential impacts of xenobiotics on wildlife species. The use of flow cytometry as an effective and rapid technique to quantify and identify immune cells has been adapted for heterophil determination. This is the first study in which that phagocytosis and oxidative burst have been used as biomarkers to determine immunological impacts of xenobiotics in waterfowl.

CHAPTER 4

DEVELOPMENTAL METHODOLOGY FOR THE EVALUATION OF IMMUNE FUNCTIONS IN LESSER SCAUP FEEDING ON ZEBRA MUSSELS FROM POLLUTED WATER.

4.1 Abstract

We assessed the influence of a diet of zebra mussels (*Dreissena polymorpha*) from Lake Erie and the St.-Lawrence river, on the immune system of lesser scaup (*Aythya affinis*). The Great Lakes-St. Lawrence River basin is among the most contaminated water bodies found in Canada. Zebra mussels rapidly accumulate high levels of polyhalogenated hydrocarbons and heavy metals. These toxicants may have deleterious health consequences on lesser scaup which feed exclusively on zebra mussels for several months of the year. The immune system, with its capacity to destroy foreign particles and protect the host against diseases, can serve as a useful sentinel of the health status of environmentally stressed organisms. Immune parameters of lesser scaup fed with zebra mussels from Lake Erie, the St.-Lawrence River or a contaminant-free diet (control) were evaluated. Phagocytic activity and oxidative burst of heterophils were evaluated by flow cytometry. After 6 weeks on the two zebra mussel diets, there was a trend toward lower phagocytic activity and bactericidal potential when compared to the control group. This may have resulted in the

pathological problems encountered in exposed groups at the end of the experiment. Birds from the St. Lawrence River group (40%) and Lake Erie group (40 %) had variable degrees of pododermatitis (bumble foot) compared to the control group, where no case of this bacterial infection was detected. In conclusion, flow cytometry assays of phagocytosis and oxidative burst can serve as biomarkers of the immune status of birds and provide important information for evaluating the health of birds exposed to environmental contamination.

4.2 Introduction

Bioaccumulation in the aquatic food chain results in high concentrations of many contaminants in organisms occupying upper trophic levels. Following bioaccumulation, xenobiotics such as heavy metals, polyhalogenated aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) can influence many physiological processes. For example, liver enzyme induction (Bosveld and Van den Berg, 1994), embryotoxicity (Tillit *et al.*, 1992), skeletal deformities (Gilbertson *et al.*, 1991) and reduced parental attentiveness (Fox *et al.*, 1978) were observed in birds inhabiting and feeding in relatively contaminated areas of North America. Among the broad range of physiological processes affected by contaminants, the immune system has been shown to be adversely affected in laboratory birds (Grasman and Scanlon, 1995). Immunodepression in some cases led to an increased susceptibility to infections (Friend and Trainer, 1974). Little information is available regarding immunotoxic effects of bioaccumulated toxicants in wildlife species (Swart *et al.*, 1994).

Since their introduction in the late 1980's, zebra mussels (*Dreissena polymorpha*) have invaded a great deal of eastern North America's waters. Due to their high filtering capacity and lipid content, this mollusk can bioaccumulate large amount of xenobiotics (Mersch *et al.*, 1993; Kraak *et al.*, 1994; Marvin *et al.*, 1994). Lesser scaups (*Aythya affinis*) are important predators of contaminated zebra mussels (Wormington and Leach, 1992; Hamilton *et al.*, 1994;. Mazak, 1995). We used lesser scaup as a high trophic level species

model to assess biochemical and physiological impacts of *Dreissena* via food chain transfer (Chapter 5). Wild waterfowl may be especially vulnerable to immunotoxicant enhancement of disease because they feed in areas where toxicants tend to accumulate, and they are gregarious animals which facilitate the spread of pathogens (Rocke *et al.*, 1984).

Heterophils are an important part of the avian immune system and function as the first line of defense against invading agents, especially bacteria (Powell, 1987). Ingestion of foreign material through the process of phagocytosis, and its destruction through the production of reactive oxygen species (ROS), known as the respiratory burst, are the major functions of heterophils. Estimation of the activity of heterophils may be a valuable tool to document the influence of environmental factors on resistance to infection.

Traditional methods for quantification of phagocytosis (e.g. microscopic visualization) require considerable effort and are susceptible to experimental error. Radioactive tracers are in widespread use but this method cannot discriminate phagocytic activities among individual cells. The different techniques for evaluating the oxidative burst such as monitoring the superoxide anion, and fluorometric and spectrofluorometric methods designed to quantify hydrogen peroxide production, present similar problems.

Since isolation of pure heterophils from blood is time consuming and may activate cells, we adopted a method that employs a one-step blood leukocyte isolation procedure for determining phagocytosis and oxidative burst. Due to the differences in light scattering of the cells, flow cytometric analysis allows the

evaluation of the status of single cells. By means of flow cytometry (FCM) it is possible not only to determine the proportion of phagocytic cells or responsive cells but also to measure the phagocytic activity and the level of production of H_2O_2 of individual cells from the same population.

The assays of immune function reported here were adapted and tested for their potential use as biomarkers of waterfowl having been exposed to environmental contamination through their diet. In order to assess the impact of chronic contaminant exposure on the immune system of lesser scaup, we conducted a six-week study under semi-natural conditions. Three groups of lesser scaup were fed with either contaminant-free duck chow, or zebra mussels from contaminated sections of western Lake Erie or the St. Lawrence River. Blood samples were collected at regular intervals and phagocytosis and respiratory burst determined.

4.3 Materials and Methods

4.3.1 Animals and diet

Lesser scaup eggs from six different nests were collected in St. Denis near Saskatoon on July 29, 1994. Eggs were artificially incubated and brooded at the Avian Science and Conservation Centre, Macdonald Campus, McGill University, Ste. Anne de Bellevue (Chapter 3). Ducklings were fed Duck-starter diet (Nutribec # 4281, Québec, Canada) *ad libitum*.

Three groups of 10 randomly chosen ducks (control, St. Lawrence River and Lake Erie) were transferred when 3 weeks old to external pens where they were acclimatized for a period of 18 days before feeding trials began.

Zebra mussels were collected from the Port of Montreal in the St. Lawrence River, and off Middle Sister Island, in the western basin of Lake Erie by scuba divers. Mussels were kept frozen in polypropylene bags until given to the lesser scaup.

Diets were designed to assess the potential nutritive value and contamination burden of zebra mussel as a food source for lesser scaup. In Lake Erie today, zebra mussels constitute the main food item in the diet of lesser scaup (Mazak, 1995). Therefore, lesser scaup from Lake Erie group received 5 kg of mussels per day and 500g of duck chow (Nutribec #4282, Québec, Canada), while the St. Lawrence River group received 7.5 kg of mussels plus 400g of duck chow. The control group was fed duck chow *ad libitum*.

Blood sampling was done the day before feeding trials began and at 3 week intervals thereafter. General macroscopic examinations to detect any gross pathological impairment were done after each sampling.

Organic contaminant profiles in the livers of lesser scaup were determined at the end of the experiment. Forty-three PCB congeners were detected and averaged 0.51 ppb, 14.76 ppb and, 51.95 ppb total PCBs for the control, St. Lawrence River and Lake Erie groups, respectively. This trend correlated with the contaminant burden analyzed in whole zebra mussels (Chapter 5).

4.3.2 Blood sampling and isolation of leukocytes

Leukocytes were isolated prior to the evaluation of phagocytosis and respiratory burst. Blood samples were collected via brachial venipuncture. Heterophils were isolated from heparinized blood by the following procedure: Ficoll-Paque (1ml) was layered on top of 3 ml of Mono-Poly resolving medium (3ml) in a sterile tube. Whole blood (2 ml) was then layered on top of this cushion. The tubes were centrifuged for 20 min (room temperature) at 600 x g. The heterophil layer was aspirated and cells were washed twice at 500 x g for 10 min. The final pellet was resuspended in a calcium-free, magnesium-free, phosphate-buffered saline (PBS) containing 5 mM of d-glucose (PBSg).

4.3.3 Phagocytosis

For the evaluation of phagocytosis, cell concentration was adjusted to 2.0×10^6 leukocytes/ml. Three ml of the cell suspensions were put into tubes either on ice and used as negative controls, or in a water bath at 37°C under agitation. After 30 min, 1 μ m diameter fluorescent latex beads (Molecular probes, Eugene, Oregon, U.S.A.) were added to the cell suspensions at ratios of 100 beads per leukocyte. At different intervals, 500 μ l of each cell suspension (on ice and 37°C) was layered on a cushion of 3% bovine serum albumin gradient and centrifuged at 150 g for 8 min at 4°C. The supernatant containing the free beads was withdrawn and 1 ml of an isotonic solution (Hematall, Fisher Scientific, Pittsburg, Pennsylvania, U.S.A.) was added to the remaining cells. The fluorescence of approximately 10,000 cells was read with a FACScan (Becton

Dickinson, Rutherford, New Jersey, U.S.A.) flow cytometer adjusted with a threshold on the forward scatter to avoid contamination with cell debris or erythrocytes; the vast majority of the events recorded in this manner are leukocytes.

4.3.3 Respiratory burst

The intracellular production of ROS in stimulated heterophils was quantified in individual cells by flow cytometry using a modification of the procedure reported by Bass and co-workers (1983). Cells were adjusted to a concentration of 2.0×10^6 leukocytes/ml in a 1 g/L PBSg. One ml of cell suspension was incubated 15 min at 37°C, in the dark, with 5 μ M of 2,7-dichlorofluorescein diacetate (DCFDA, Molecular Probe, Eugene Oregon, U.S.A.), a probe used to quantify the production of H₂O₂. Thereafter, the labelled cells were kept in the dark before addition of the stimulus: 100 nM of phorbol 12-myristate 13-acetate (PMA). Control samples containing no stimulus were run in parallel; the mean fluorescence of the unstimulated cells (no PMA) was subtracted from the mean fluorescence of the cells activated with PMA. The fluorescence of approximately 10,000 cells was measured with a FACScan (Becton Dickinson, Rutherford, New Jersey) flow cytometer, using the LYSYS-II software.

4.3.5 Statistical analyses

Mann and Whitney U tests (SYSTAT software) were used to detect differences between exposed and control groups. Differences between groups were considered significant at $p < 0.05$. Test power and estimation of required sample sizes to detect significant differences given observed variability were determined following the methodology of Rosner (1995). For sample size estimation, a two-sided test with significance level $\alpha = 0.05$ and power = 0.8 was used. Among-group relative variability was computed following Lewontin (1966).

4.4 Results

4.4.1 Adaptation of the FCM immunological assay

A scattergram of the flow cytometric profile of the separated peripheral blood leukocytes is shown in Fig. 4.1. Cells are displayed according to their size (FSC; x axis) and complexity (SSC; y axis). Purified leukocytes showed some contaminated lymphocytes which were rather small cells with low complexity, while heterophils are clearly more complex or granular. Region R1 was drawn to select heterophils. In setting the region R1, we must keep in mind that activation of cells in phagocytosis or PMA activation will result in a modification of FSC and SSC properties. In the analyses, the correct gate setting was adjusted, as shown in Fig 4.1A. After the region was set, the different fluorescences were displayed. Fig. 4.1B illustrates the green fluorescence of stimulated cells with PMA.

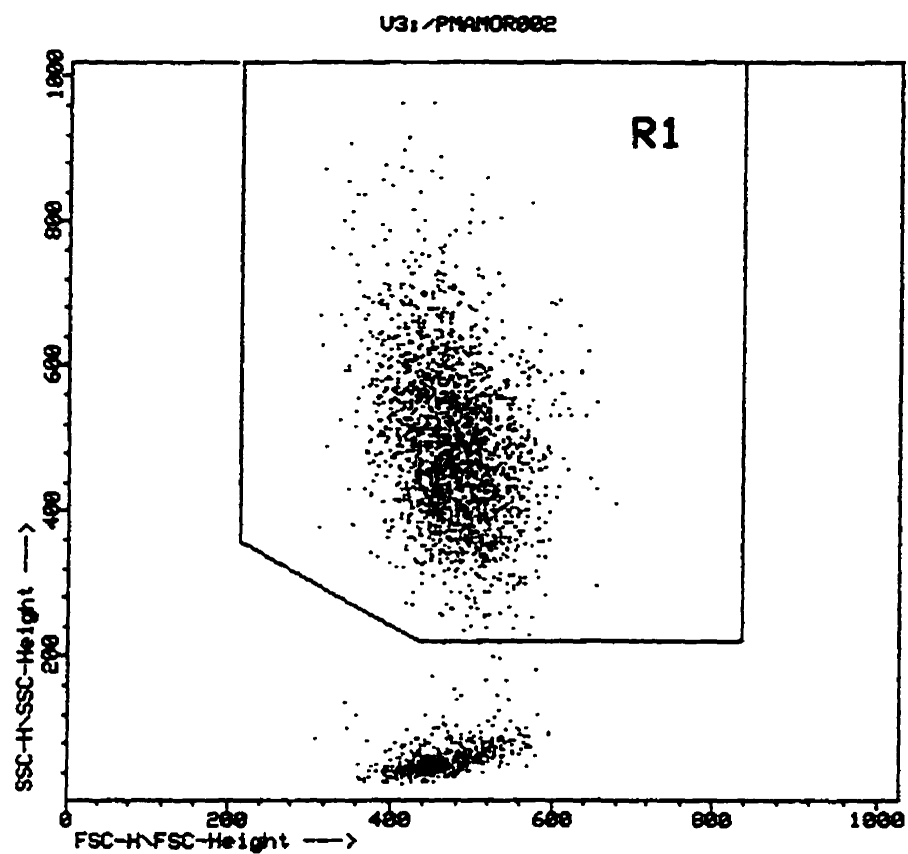


Figure 4.1 A. Scattergram of the flow cytometry profile of isolated peripheral blood before stimulation with PMA.

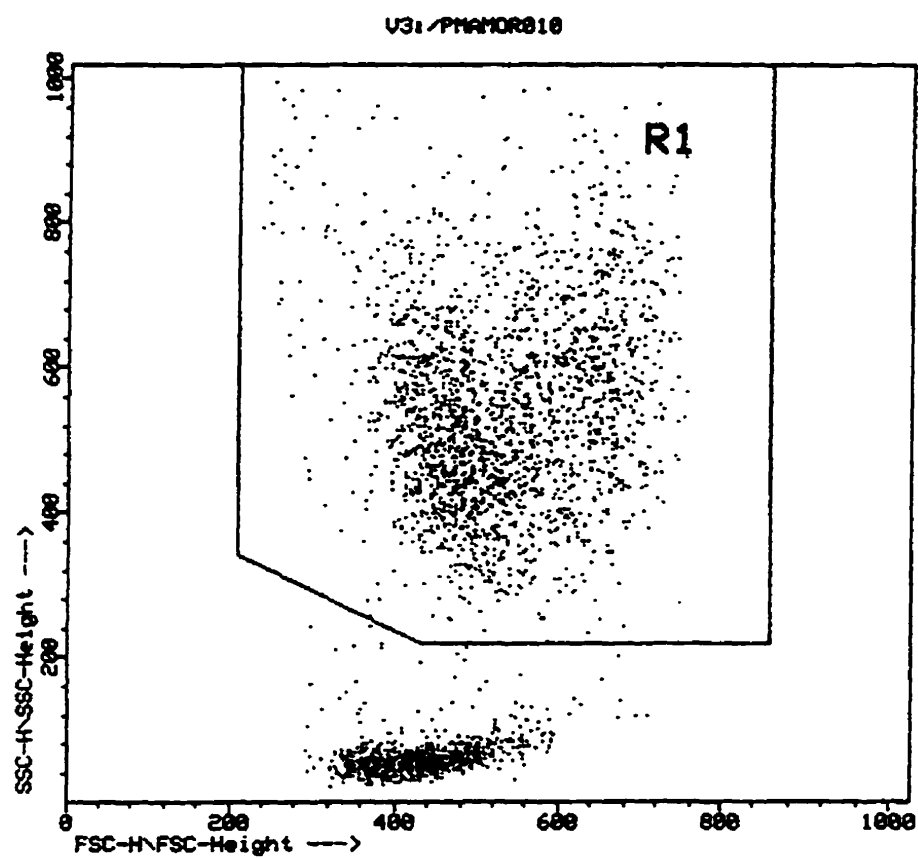


Figure 4.1 B. Scattergram of the flow cytometry profile of isolated peripheral blood after stimulation with PMA.

Approximately 95% of heterophils responded to PMA stimulation.

We performed a time-response curve to determine maximum H_2O_2 response by cells treated with PMA (Fig 4.2). In these experiments, peak response was observed at 30 min, and thereafter plateaued and remained steady until 60 min. Therefore, a 30 min incubation period was chosen for the measurement of the oxidative burst.

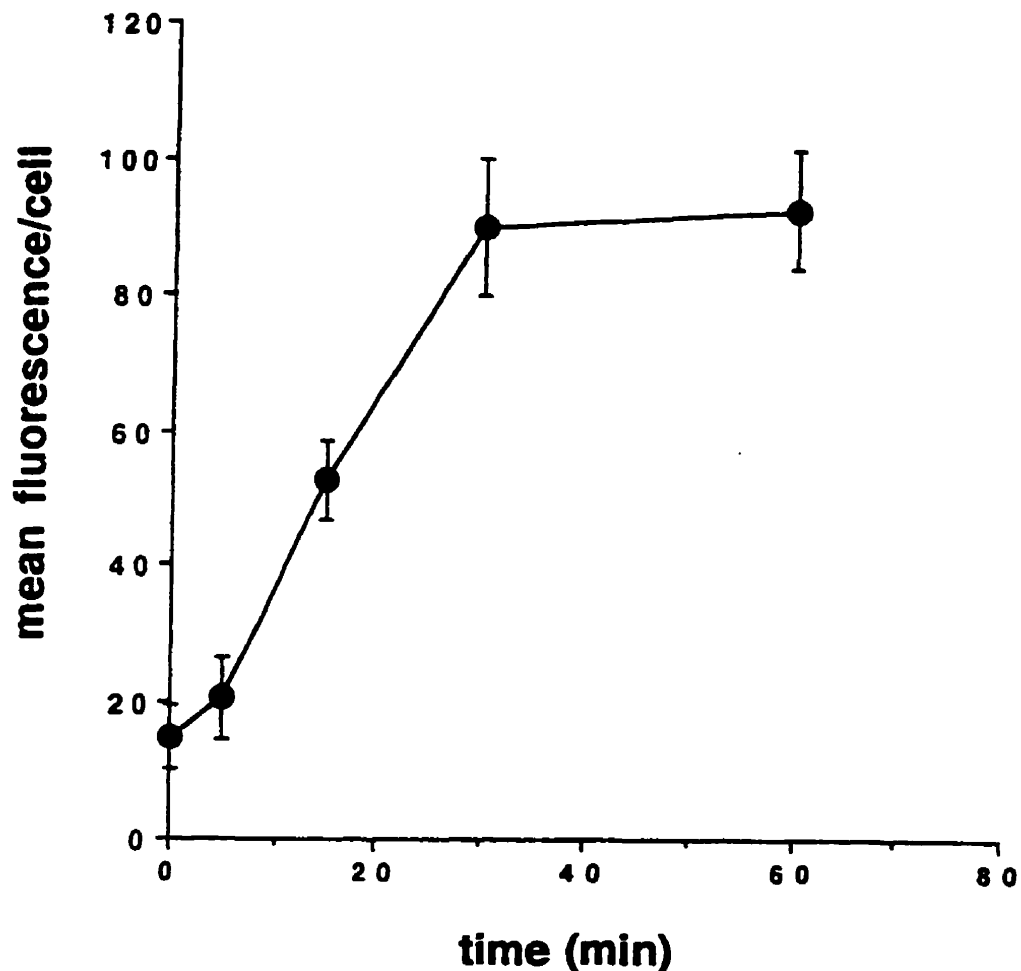


Figure 4.2. Time course of oxidative burst in PMA stimulated heterophils. Values are means (\pm standard deviation) of fluorescence per cell.

4.4.2 Phagocytosis

We observed in the St. Lawrence River lesser scaup group an initial increase in the number of phagocytic cells after 3 weeks, followed by a steep decrease (31.15%) relative to the control group at week 6 ($p > 0.05$). This trend was not as strong for the Lake Erie group (Fig. 4.3).

A different pattern was observed for the capacity of heterophils to phagocyte fluorescent microspheres. Initially, the phagocytic capacity increased at week 3 for the Lake Erie group, followed by a drop of 26% compared to controls at week 6 ($p > 0.05$). The phagocytic capacity of birds from the St. Lawrence River group continuously and steadily decreased to a minimum of 47.51% of controls at the end of the study ($p > 0.05$) (Fig. 4.4).

The lack of significance may be explained by small sample sizes and high variability within each group. Power analyses indicated that 40 ducks per group would have been required to detect significant phagocytic immunosuppression at week six in exposed groups.

4.4.3 Oxidative Burst

Oxidative burst activity decreased steeply with time for the St. Lawrence River group (Fig. 4.5) compared to the control. Peroxide production from the St. Lawrence River group was diminished by 53% compared to the controls at the end of the study. After 6 weeks of eating mussels, Lake Erie scaup had a reduction of 26%, in their capacity to produce peroxide products compared to the control group. However, these reductions were not significantly different from the

control group. Again, the lack of significance may have been due to small sample size (n is between 5 and 9), coupled with increasing variability in the highest contaminant exposed group. Thus, the within group variability for the Lake Erie group was significantly higher than that observed for the controls ($F = 11.12$, $p < 0.025$).

4.4.4 Pododermatitis incidence

Pododermatitis (bumble foot) was encountered in 40% of both the St. Lawrence and the Lake Erie groups. Two birds from the Lake Erie group with the lowest phagocytic and oxidative burst activities had severely infected foot erosions. No pododermatitis occurred in the control group.

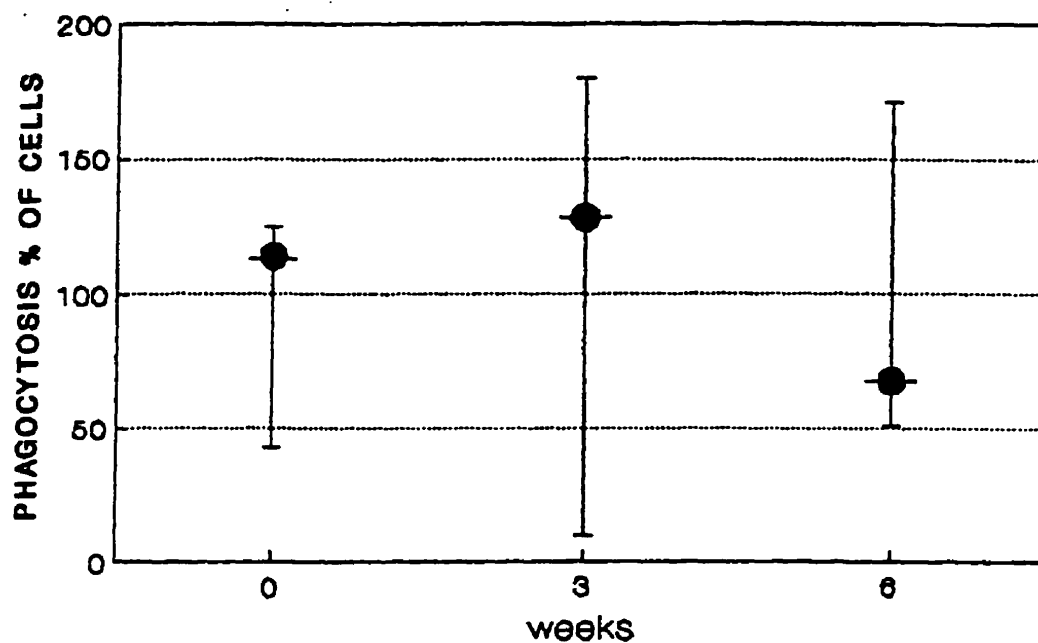
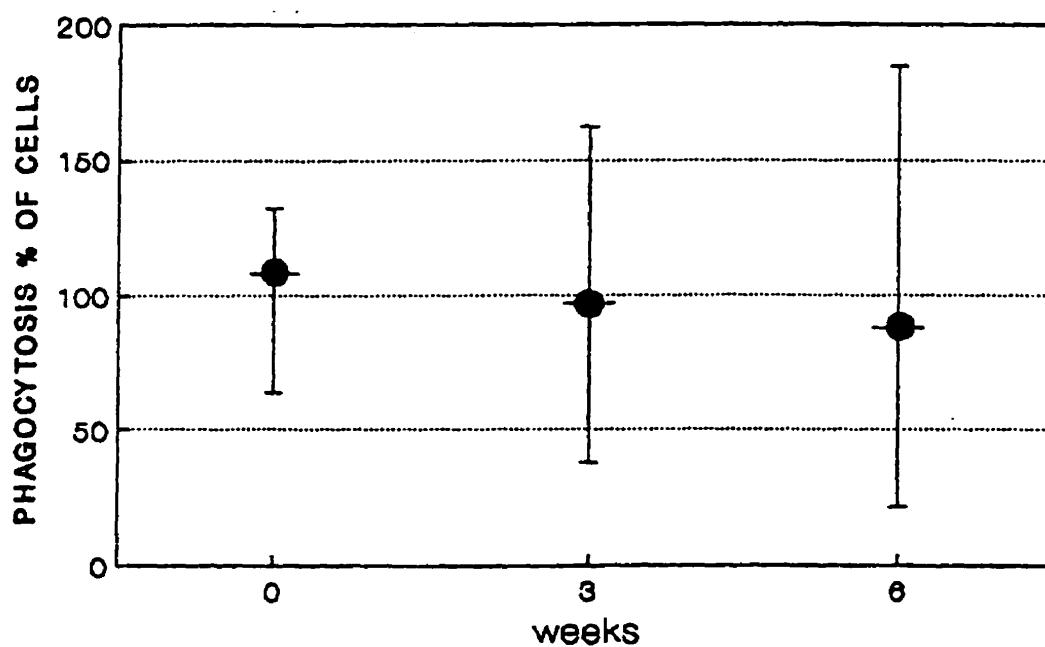
A**B**

Figure 4.3. Number of phagocytosing cells in lesser scaup fed with zebra mussels from the St-Lawrence River (A) or from Lake Erie (B). The results are expressed as a percentage of the response observed by birds fed the control diet. Circles are medians and the bars give ranges.

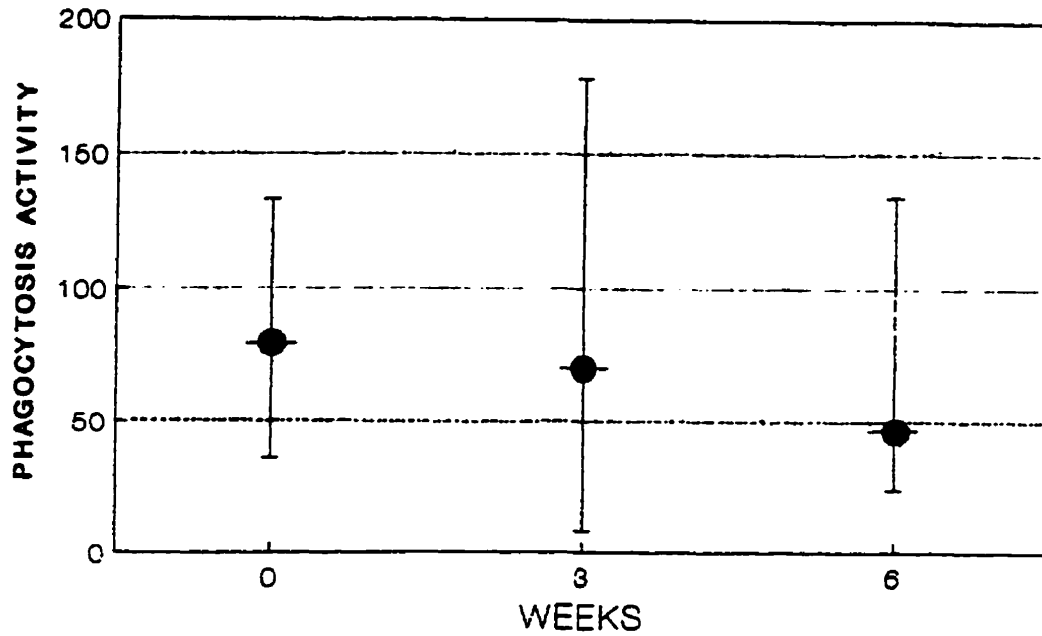
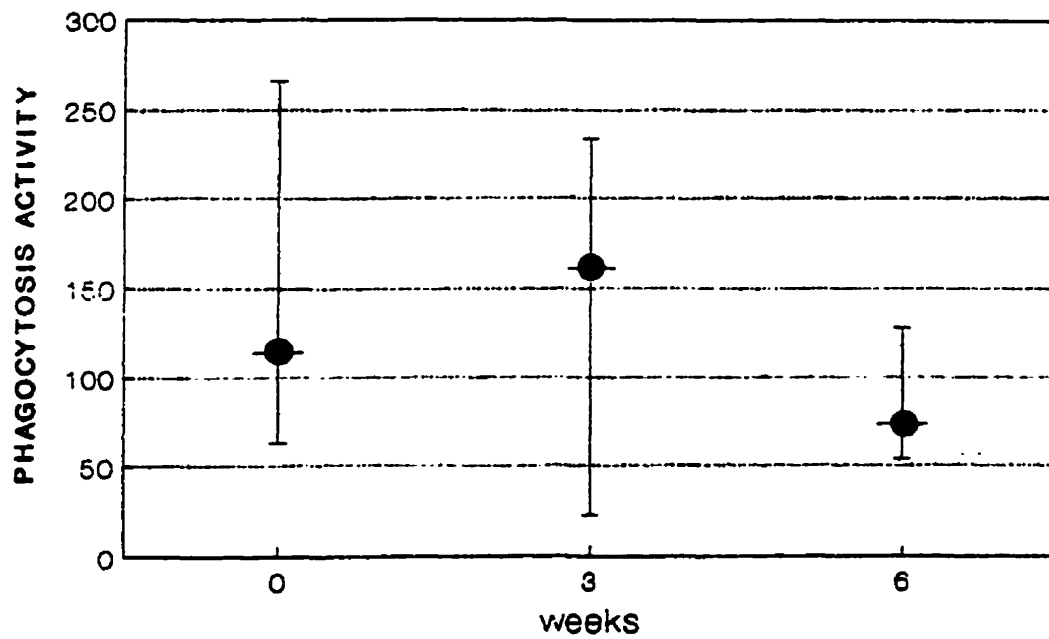
A**B**

Figure 4.4. Phagocytosis activity of heterophils from lesser scaup fed with zebra mussels from the St-Lawrence River (A) or from Lake Erie (B). The results are expressed as a percentage of the response observed by birds fed the control diet. Circles are medians and the bars give ranges.

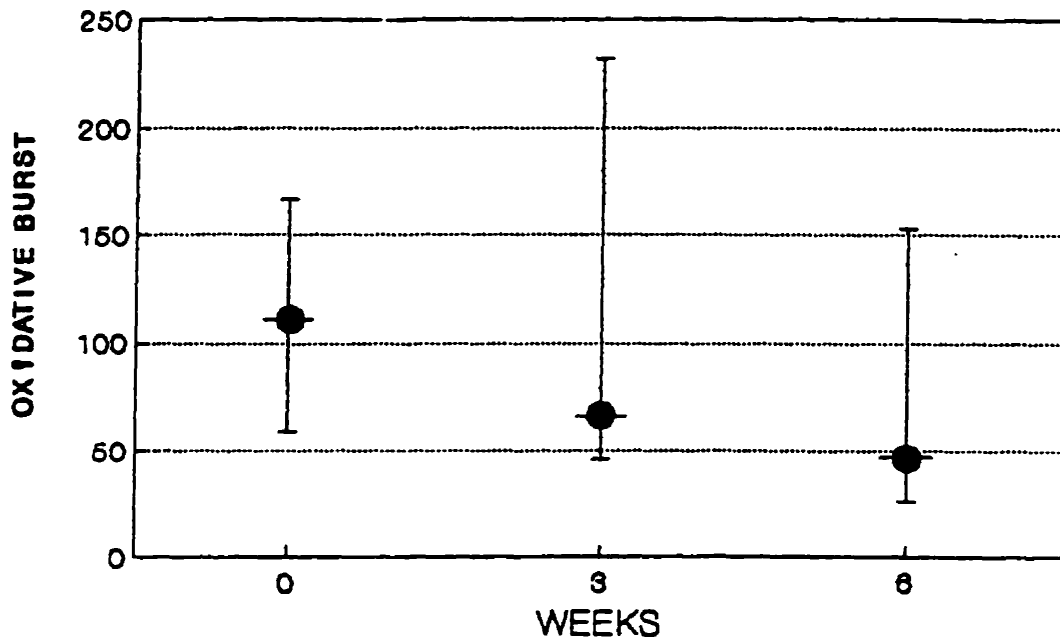
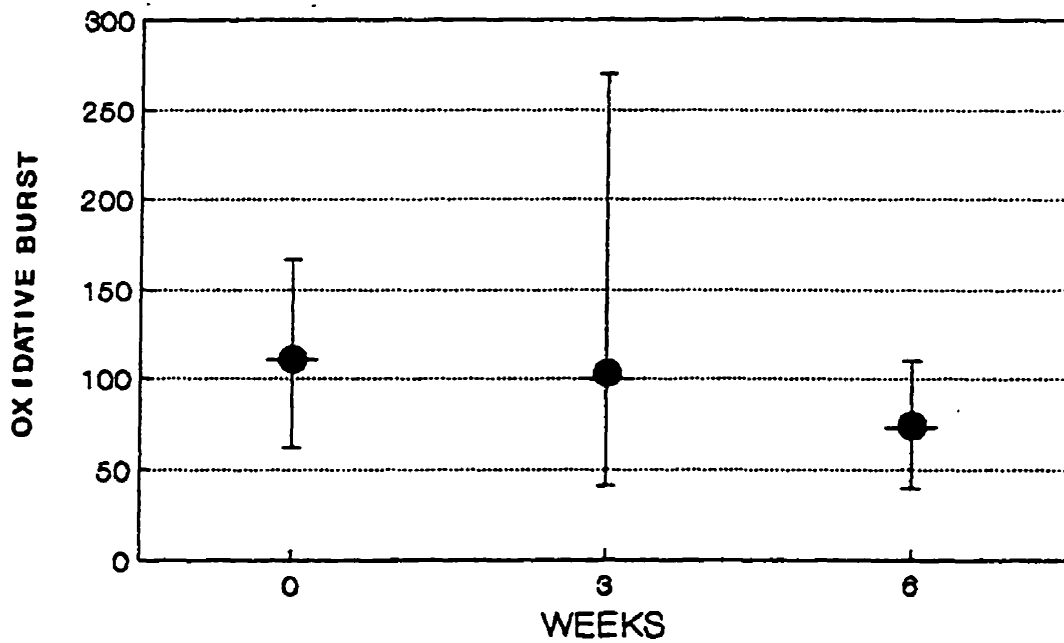
A**B**

Figure 4.5. Oxidative burst activity of heterophils from lesser scaup fed with zebra mussels from the St-Lawrence River (A) or from Lake Erie (B). The results are expressed as a percentage of the response observed by birds fed the control diet. Circles are medians and the bars give ranges.

4.5 Discussion

Several authors, using different methods, have evaluated the effects of xenobiotics on phagocytic and respiratory burst activities in neutrophils. For example, dieldrin exposure reduces phagocytic (Krzystyniak *et al.*, 1985) and respiratory burst (Krzystyniak *et al.*, 1987) activities. Phagocytic activity was also influenced by exposure to mercury (Brunet *et al.*, 1993), cadmium (Loose *et al.*, 1978) and lead (Tam and Hindhill, 1984). Respiratory burst activity was affected by exposure to cadmium (Guillard and Lauwerys, 1989), lead (Buchmüller-Rouiller *et al.*, 1989) and mercury (Guillard and Lauwerys, 1989; Contrino *et al.*, 1992). However, difficulties arise when applying these tests to wild animals exposed to environmental stresses under natural conditions.

The problems associated with the isolation and purification of different cell types in a new species were avoided by using flow cytometry. With this technique, different cell populations can easily be differentiated and gated according to their size and complexity. Other advantages of this approach are the need for relatively few cells, and the opportunity to obtain rapid and consecutive measurements using the same specimen. Flow cytometry also permits measurement of 10 000 cells over a short period of time. To optimize conditions for phagocytic assay, we chose fluorescent latex bead as the material to be phagocytized. The fluorescence emitted from these particles is homogenous and easily quantified by flow cytometry. Furthermore, inert latex beads are not degraded after phagocytosis and the phagocytic activity is not

influenced by previous exposure of the bird to any antigen as could occur in assays based on bacterial ingestion.

Our flow cytometry results confirm the involvement of oxidative process in heterophils after stimulation with PMA. Optimal conditions to monitor cell stimulation included a DCFDA leading concentration of 5 μ M and a 20 min incubation with soluble activator PMA. Shifts in fluorescence have revealed that heterophils respond uniformly as a single population. These data are comparable with earlier observations (Bass *et al.*, 1983) using DCFDA stained human neutrophils activated with PMA. We also demonstrated that heterophils alter their light scattering properties following PMA activation, changing to relatively larger cells. The FSC shift may be due to the degranulation associated with cell activation. Similar changes in scattergrams were observed with neutrophils undergoing oxidative burst in different species (Perticarari *et al.*, 1994; De Guise *et al.*, 1995).

The second part of this study was to validate these techniques using lesser scaup fed contaminated zebra mussels. A decrease in the phagocytosis and oxidative burst activities in exposed animals was observed. Although these results did not significantly differ from controls, they correlated well with the pathology of exposed groups at the end of feeding trials. Indeed, birds from the St. Lawrence River group (40%) and Lake Erie group (40%) demonstrated variable degrees of pododermatitis compared to controls where no bumble foot was detected. The higher incidence of pododermatitis in lesser scaup groups feeding on zebra mussels may be explained by the decrease in both phagocytic

capacity and peroxidic activity. Thus, individual susceptibility to infection by the microorganisms that caused the bumble foot was inversely proportional to both phagocytic capacity and oxidative burst response. Several studies had demonstrated that PCB-treated animals were more susceptible to viral and bacterial infections (Koller, 1979; Faith *et al.*, 1980).

This study was not designed to discriminate between zebra mussel xenobiotic burden and nutritive deficiencies in the different groups. In spite of that, one can speculate that pododermatitis may result from the decrease in phagocytosis and oxidative burst responses which may be caused by direct effect of contaminant on the immune system, or indirectly, by nutritional deficiency (particularly vitamin A) (Wobeser and Kost, 1992).

Variability in both functions was rather large. Although this might be due to the small size of the sample, the variability in the evaluation of phagocytosis and respiratory burst in other species was also relatively high (Lindena and Burkhardt, 1988; Trowald-Wigh and Thoren-Tolling, 1990; Daley *et al.*, 1991; Hallen Sandgren *et al.*, 1991)

Stressed individuals within a group are expected to respond with greater variability than individuals not submitted to the same stressor. In this study, we observed that the within group variability was significantly greater in the Lake Erie group than in the controls. Great variability within groups in immunological responses was also reported in other birds exposed to anthropogenic stress (Trust *et al.*, 1990, 1994).

The lack of statistical difference between exposed and control groups may be due to either small sample size/great variability or to the absence of immunological effects resulting from zebra mussel ingestion. However, the presence of pododermatitis only in ducks fed zebra mussels suggests an impairment of their health. Concomitant with the use of immunological responses, other biochemical biomarkers were assessed and significant differences were observed in vitamin A concentration, porphyrin accumulation, and hepatocyte histological analyses (Chapter 5). Therefore, we presume that the immunological impacts were real, but our small sample sizes reduced our statistical power. Thus, increasing the number of individuals per group would probably lead to statistically significant immunosuppression in both phagocytosis and oxidative burst activities. Other studies are needed to better understand the immunotoxicological impacts of chronic exposure to environmental contaminants accumulated through the food chain.

These assays may serve as useful biomarkers of the immune status and can give important information for evaluating the health of birds exposed to environmental contamination.

4.6 References

- Bass, D.A., J.W. Parce, L.R. Dechatelet, P. Szejda, M.C. Seeds, and M. Thomas. 1983. Flow cytometric studies of oxydative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* 130:1910-1917.
- Bosveld, A. and M. van den Berg. 1994. Effects of polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and dibenzofuran (PCDFs) on fish eating birds. *Environ. Rev.* 2:147-166.
- Brunet, S., F. Guertin, D. Flipo, M. Fournier, and K. Krzystyniak. 1993. Cytometric profiles of bone marrow and spleen lymphoid cells after mercury exposure in mice. *Int. J. Immunopharmac.* 15:811-819.
- Buchmüller-Rouiller, T., A. Rasijn, and J. Mauël. 1989. Lead inhibits oxidative metabolism of macrophages exposed to macrophage-activating factor. *Biochem. J.* 260:325-332.
- Contrino, J., L.L. Kosuda, P. Marucha, D.L. Kreutzer, and P.E. Bigazzi. 1992. The in vitro effects of mercury on peritoneal leucocytes (PMN and macrophages) from inbred Brown Norway and Lewis rats. *Int. J. Immunopharmacol.* 14:1051-1059.
- Daley, M.J., E.R. Oldham, T.J. Williams, and P.A. Coyle. 1991. Quantitative and qualitative properties of host polymorphonuclear cells during experimentally induced *Staphylococcus aureus* mastitis in cows. *Am. J. Vet. Res.* 52:474-479.

- De Guise, S., D. Flipo, J.R. Bohem, D. Martineau, P. Béland, and M. Fournier. 1995. Immune function in beluga whale: evaluation of phagocytosis and respiratory burst with peripheral blood leukocytes using flow cytometry. *Vet. Immunol. Immunopathol.* 47:351-362.
- Faith, R.E., M.I. Luster, and J.G. Vos. 1980. Effects on immunocompetence by chemicals of environmental concern. *Rev. Biochem. Toxicol.* 2:173-211.
- Friend, M., and D.O. Trainer. 1974. Experimental dieldrin-duck hepatitis virus interaction studies. *J. Wildl. Manag.* 38:396.
- Gilbertson, M., T. Kubiak, J. Ludwig, and G.A. Fox. 1991. Great Lake embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. *J. Tox. Env. Health* 33:455-520.
- Grasman, K.A., and P.F.S. Scanlon. 1995. Effects of acute lead ingestion and diet on antibody and t-cell-mediated immunology in Japanese quail. *Arch. Environ. Contam. Toxicol.* 28:161-167.
- Guillard, O., and R. Lauwerys. 1989. *In vitro* and *in vivo* effect of mercury, lead and cadmium on the generation of chemiluminescence by human whole blood. *Biochem. Pharmacol.* 38:2819-2823.
- Hallen Sandgren, C., K. Nordling, and I. Björk. 1991. Isolation and phagocytic properties of neutrophils and other phagocytes from nonmastitic bovine milk. *J. Dairy Sci.* 74:2965-2975.
- Hamilton, D.J., C.D. Ankney, and R.C. Bailey. 1994. Predation of zebra mussels by diving ducks: an enclosure study. *Ecology* 75:521-531.

- Koller, L.D. 1979. Effects of environmental contaminants on the immune system. *Adv. Vet. Sci. Comp. Med.* 23:267-295.
- Kraak, M.H.S., Y.A. Wink, S.C. Stuijzand, M.C. Buckert-de Jong, C.J. de Groot, and W. Admiral. 1994. Chronic ecotoxicology of Zn and Pb to the zebra mussel *Dreissena polymorpha*. *Aquat. Toxicol.* 30:77-89.
- Krzystyniak, K., B. Trottier, P. Jolicoeur, and M. Fournier. 1987. Macrophage functional activities versus cellular parameters upon sublethal exposure in mice. *Mol. Toxicol.* 1:247-259.
- Krzystyniak, K., P. Hugo, D. Flipo, and M. Fournier. 1985. Increased susceptibility to mouse hepatitis virus 3 of peritoneal macrophages exposed to dieldrin. *Toxicol. Appl. Pharmacol.* 80:397-408.
- Lewontin, R.C. 1966. On the measurement of relative variability. *Systematic Zool.* 15:141-142.
- Lindena, J., and H. Burkhardt. 1988. Separation and chemiluminescence properties of human, canine and rat polymorphonuclear cells. *J. Immunol. Meth.* 115:141-147.
- Loose, L.D., J.B. Silkworth, and D.W. Simpson. 1978. Influence of cadmium on phagocytic and microbicidal activity of murine peritoneal macrophages, pulmonary alveolar macrophages, and polymorphonuclear neutrophils. *Infect. Immun.* 22: 378-381.
- Marvin, C.H., B.E. McCarry, and D.W. Bryant. 1994. Determination and genotoxicity of polycyclic aromatic hydrocarbons isolated from *Dreissena*

- polymorpha* (zebra mussels) from Hamilton Harbour. J. Great Lakes Res. 20:523-530.
- Mazak, E.J. 1995. Organic contaminants in lower Great Lakes' waterfowl in relation to diet with particular reference to *Dreissena polymorpha*. M.Sc. Thesis, University of Windsor. 111 pp.
- Mersch, J., E. Morhain, and C. Mouvet. 1993. Laboratory accumulation and depuration of copper and cadmium in the freshwater mussel *Dreissena polymorpha* and the aquatic moss *Rhynchostegium riparioides*. Chemosphere 27:1475-1485.
- Perticarari, S., G. Presani, and E. Banfi. 1994. A new flow cytometric assay for the evaluation of phagocytosis and the oxidative burst in whole blood. J. Immunol. Methods 170:117-124.
- Powell, P.C. 1987. Macrophages and other nonlymphoid cells contributing to immunity. In: T.Toivanen and P.Toivanen (Eds). Avian immunology: basis and practice. CRC Press, Boca Raton, Florida, pp. 195-212.
- Rocke, T.E., T.M. Yuill, and R.E. Hinsdill. 1984. Oil and related toxicant effects on mallard immune defenses. Environ. Res. 133:343-352.
- Rosner, B. 1995. Fundamentals of Biostatistics. Wadsworth Publishing Co. Belmont, CA. 682 pp.
- Swart, R. L., P.S. Ross, L.J. Vedder, H.H. Timmerman, S. Heisterkamp, H. Van Loveren, J. G. Vos, J.H. Reijnders, and A.D. Oterhaus. 1994. Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. Ambio 23:155-159.

- Tam, P.E., and R.D. Hinsdill. 1984. Evaluation of immunomodulatory chemicals: alteration of macrophage function in vitro. *Toxicol. Appl. Pharmacol.* 76:183-194.
- Tillit, D.E., G.T. Ankley, J.P. Giesy, H. Kurita-matsuba, D. Weseloh, P.S. Ross, C. Bishop, L. Sileo, K. Stromborg, J. Larson, and T.J. Kubiak. 1992. Polychlorinated biphenyl residues in eggs and mortality in double-crested cormorants from the Great Lakes. *Environ. Toxicol. Chem.* 11:1281-1288.
- Trowald-Wigh, G., and K. Thoren-Tolling. 1990. Phagocytosis induced by yeast cells in canine granulocytes: a methodological study. *Acta Vet. Scand.* 31:87-95.
- Trust, K.A., A. Fairbrother, and M.J. Hooper. 1994. Effects of 7,12-dimethylbez[a]anthracene on immune function and mixed-function oxygenase activity in the European starling. *Environ. Toxicol. Chem.* 13:821-830.
- Trust, K.A., M.W. Miller, J.K. Ringelman, and I.M. Orme. 1990. Effects of ingested lead on antibody production in mallards (*Anas platyrhynchos*). *J. Wildl. Dis.* 26:316-322.
- Vos, J.G. 1977. Immune suppression as related to toxicology. *CRC Crit. Rev. Toxicol.* 5:67-101.
- Wobeser, G., and W. Kost. 1992. Starvation, staphylococcosis, and vitamin A deficiency among mallards overwintering in Saskatchewan. *J. Wildl. Dis.* 28:215-222.

Wormington, A. and J.H. Leach. 1992. Concentrations of migrant diving ducks at Point Pelee National Park, Ontario, in response to invasion of zebra mussels, *Dreissena polymorpha*. Can. Field-Nat. 106:376-380.

CONNECTIVE STATEMENT LINKING CHAPTERS 4 AND 5

The lowering of phagocytic activity and bactericidal potential of heterophils after 6 weeks of zebra mussel diet may explain such pathologies as pododermatitis. To further our understanding of the impacts of contaminated zebra mussels on the lesser scaup health, the next chapter reports on a series of biochemical (porphyrin profile, ethoxyresorufin-*o*-deethylase activity, vitamin A concentrations) and histopathological biomarkers experiments.

CHAPTER 5

THE ECOTOXICOLOGICAL SIGNIFICANCE OF ZEBRA MUSSELS AS A FOOD SOURCE FOR LESSER SCAUPS.

5.1 Abstract

The potential ecotoxicological impacts on lesser scaup (*Aythya affinis*) of a new, supra abundant food source, the zebra mussel (*Dreissena polymorpha*) was assessed. Three groups ($n=10$) of lesser scaup were fed zebra mussels from western Lake Erie, the St. Lawrence River or a control contaminant-free diet, respectively, for a period of 6-9 weeks. A series of biochemical and physiological biomarkers were then used to document health impairments in response to the contaminated food source. This approach was assessed to validate the use of laboratory biomarkers in organisms living in natural environments.

At the study's end, total PCBs averaged 57, 485, and 1600 $\mu\text{g/kg}$ lipid wet weight, respectively, in livers of control, St. Lawrence River and Lake Erie lesser scaup groups. Biomagnification factor values (BMF) for most PCB congeners ranged from 2-3. Fat infiltration and glycogen loading in hepatocytes were detected in St. Lawrence River and Lake Erie lesser scaup groups, respectively. Significant decreases in hepatic retinol and retinyl palmitate concentrations, and liver:body mass ratios were observed in lesser scaup fed on zebra mussels

compared to the control group. Hepatic ethoxyresorufin-*o*-deethylase (EROD) activity and porphyrin profiles were highly variable and were not significantly different among groups. The use of histopathology and vitamin A concentrations as biomarkers shows promise for monitoring “early” injury at the cellular and biochemical level by organochlorines and may help in explaining the health impairment of different species of waterfowl exposed to xenobiotics via contaminated food source.

5.2 Introduction

The zebra mussel (*Dreissena polymorpha*) is eaten by benthivorous fish and by waterfowl (Prejs *et al.*, 1990; Stanczykowska *et al.*, 1990; Hamilton, 1992; French III and Bur, 1993). Fish with pharyngeal teeth such as the European roach (*Rutilus rutilus*) and the freshwater drum (*Aplodinotus grunniens*) are able to crush zebra mussel shells. In Europe, birds such as the tufted duck (*Aythya fuligula*), pochard (*A. ferina*), common goldeneye (*Bucephala clangula*), coot (*Fulica atra*), and the greater and lesser scaups (*A. marila* and *A. affinis*) feed intensively on mussels (Géroudet, 1966; Schmidt, 1975; Stanczykowska 1977, Petroli, 1981; Suter, 1982; Stanczykowska *et al.*, 1990). In North America, the feeding habits and migratory patterns of some diving ducks have been altered in response to the proliferation of zebra mussels (Hamilton, 1992; Custer, 1993). For example, the number of lesser scaup off Point Pelee National Park increased dramatically from a maximum of 150 ducks observed prior 1988 to 13 500 in 1989 (one day counts; Wormington and Leach, 1992) and they remained in the vicinity much longer than they did prior to the arrival of zebra mussels (Hamilton *et al.*, 1994). Zebra mussels are now the main food of lesser and greater scaup in the western basin of Lake Erie (Mazak, 1995). *Dreissena* rapidly bioaccumulate certain organic contaminants to concentrations of up to 30 000 times greater than those found in the surrounding waters (Snyder, 1990). Given the known ability of these mollusks to accumulate lipophilic compounds, food chain transfer of contaminants could constitute a significant risk to

molluscivorous ducks (Scholten *et al.*, 1989; de Kock and Bowmer, 1993). Due to their great dispersal and high reproduction rate, zebra mussels have already invaded a high proportion of North America freshwaters (Strayer, 1991; New York Sea Grant, 1996). Thus, *Dreissena* may constitute an abundant and accessible contaminated food source for several diving duck species on wintering grounds and during migrations.

A series of biomarkers was used to test for the possible impacts of contaminated zebra mussel on the health of diving duck populations. Few studies have used several biomarkers concurrently to generate an integrated view of the nutritional and the xenobiotic impacts on living organisms (Chu *et al.*, 1994). Several biochemical markers have been extensively studied in laboratory conditions. However, an increasing dissatisfaction exists with the inability of many standard laboratory biomarker protocols to predict real-world situations (Giddings, 1986). Natural populations are exposed to a mixture of contaminants and are subjected to a multitude of stressors (Depledge *et al.*, 1993). Field conditions are more complex than those of laboratory and there is an urgent need to validate laboratory biomarkers in organisms living in natural environments (Cairns *et al.*, 1993).

The present study examined the toxicological and the nutritional impacts of a diet of zebra mussels on lesser scaup using various morphological, biochemical, and histopathological biomarkers. Porphyrin profiles, mixed-function oxidases and vitamin A are tools that are used to explore the relationship between xenobiotic exposure and impaired health. Moreover, these biomarkers

are known to be sensitive to halogenated aromatic hydrocarbons (HAH), which are the main pollutants in the natural systems we studied. Physiological and histopathological biomarkers are not contaminant-specific. However, they are indicators of the general health and stress level of organisms. Under acute organic exposure, laboratory studies have demonstrated an increase in the concentration of highly carboxylated porphyrins (Lambrecht *et al.*, 1988) and in ethoxyresorufin-*o*-deethylase (EROD) activity (Brunström, 1990), and a decrease in both retinol and retinyl palmitate concentrations (Spear *et al.*, 1989). However, few studies (e.g. Fox *et al.*, 1988; Spear *et al.*, 1990) have documented the impacts of low chronic exposure to a naturally occurring food source.

5.3 Materials and Methods

5.3.1 Animals and diets

Zebra mussels were collected from the Port of Montréal, St. Lawrence River, Québec, Canada and near Middle Sister Island, Lake Erie, Ontario, Canada. The mussels were collected at a depth of approximately 3 m by SCUBA divers, packed in 2.5 kg lots in large plastic bags and frozen at -20°C until thawed and fed to the lesser scaup.

Lesser scaup eggs from 6 different nests were collected in St. Denis near Saskatoon, Saskatchewan, Canada. Eggs were artificially incubated and brooded at the Avian Science and Conservation Centre, Macdonald Campus,

McGill University (Chapter 3). The ducklings were raised on wood shavings with Duck Starter diet (19% crude protein, Nutribec, #4281, Quebec) for the first two weeks after which they were given Duck Grower diet (17% crude protein, Nutribec, #4282) *ad libitum*.

At the age of 3 weeks, the ducklings were transferred to 3 different pens at the Ecomuseum of the St. Lawrence Valley Natural History Society in Ste. Anne de Bellevue, Québec, Canada. Three groups of 10 randomly selected ducklings (control, St-Lawrence River and Lake Erie) were acclimatized for an additional period of 18 days before feeding trials began.

The vitamin and energetic requirements for scaup were estimated from three different computations (Wallack and Boever, 1983; Snyder and Terry, 1986; Lewis *et al.*, 1987). The average energetic requirement for a duck (202 kcal/day) is based on estimates of metabolic energetic need at rest, for growth, for sustaining cold weather (environmental stress), and for daily activities. The food supplies necessary to reach this requirement were calculated for each group. Based on bomb calorimetry analysis, the energetic value of the soft tissue of zebra mussel was 25 kcal/100g wet weight. By extrapolation, a lesser scaup group would have to be fed 16.5 kg of mussels per day to meet their metabolic energy requirement. Providing this quantity of mussels was not feasible. Therefore, we compensated by supplying some Duck Grower to the St-Lawrence River and Lake Erie groups. The Lake Erie group received 5 kg of mussels per day and 480g of Duck Grower, the St-Lawrence River group, 7.5 kg of mussels

plus 400g of Duck Grower, and finally the control group was fed Duck Grower *ad libitum*.

Zebra mussel consumption (g of mussel excluding empty shells) was determined daily. Over a 6 week period, a total of 195.8 kg and over a 9 week period, a total of 189.4 kg of zebra mussels were eaten by the Lake Erie and St. Lawrence River lesser scaup groups, respectively. The 6 to 9 week duration of feeding trials mimicked the potential duration of a migration stop-over of lesser scaup at the sites where zebra mussels were collected.

General examinations and weight determinations of the ducks were done on August 30-31, September 19-20, October 10-11 and November 1-2, 1994. Microscopic examination of the feces for parasites was done every week. Lesser scaups from the Lake Erie group were sacrificed on Oct. 10-11 and all other ducks on Nov. 1-2, 1994.

5.3.2 Organochlorine determination

Five grams of shucked zebra mussels were blotted on paper towels to absorb excess water as they thawed. The byssus threads were removed and pooled soft tissues samples from the Lake Erie and the St. Lawrence River mussels were homogenized separately. Soft tissues were then ground in anhydrous sodium sulfate.

At sacrifice, 5 grams of the left lobe of the liver of each duck were wrapped in hexane-rinsed aluminum foil. Samples were kept frozen at -20°C until used. Thawed livers were ground in anhydrous sodium sulfate.

Halogenated contaminants in zebra mussel soft tissue and liver samples from lesser scaup were determined using standard isolation methods followed by separation and quantification by gas chromatography-electron capture detection at the Great Lakes Institute, University of Windsor (Lazar *et al.*, 1992). A 2 μ L sample of the solution was injected into the gas chromatograph using a H.P. 7673A auto injector. A H.P. 3396 integrator, and a gas chromatography column of 30m x 0.25 mm i.d. with 0.25 μ m DB-5 film thickness were used for the analyses.

Total PCBs were calculated using the concentration of 43 PCB congeners. Total organochlorine (OC) included the concentrations of the following pesticides: 1,2,3,4-TCB, QCB, HCB, OCS, *trans*-nonachlor, Mirex, *g*-HCH, oxy-Chlordane, DDE, DDT, DDD, *cis*-Nonachlor, HC Epox, Dieldrin. Concentrations are reported on a μ g/kg, wet weight basis.

5.3.3 Biomarker determination

Immediately after death, lesser scaup liver samples were frozen at -80°C in polyethylene containers and held at this temperature until analyzed.

Microsomal Preparation: Microsomes were isolated by gel filtration according to a modification of the method of Pyykko (1983). The gel suspension (Sephacrose CL-28) was poured into small glass columns (7mm i.d. X 20 cm) and packed to a volume of 4 ml. The liver was rinsed three times with an ice-cold 0.1 M sodium phosphate buffer, pH 7.4 and chopped with scissors into small pieces. All subsequent operations were carried out at -4°C. Two hundred mg of minced

liver were transferred into a 2 ml Potter-Elvehjem tissue grinder and homogenized with 800 μ l of 0.1 M sodium phosphate buffer, pH 7.4, using 10 down-and-up strokes of a hand operated Teflon pestle. The homogenate was transferred to a 1.8 ml microtube and centrifuged at -4°C for 15 min at 9,000 x g. Without disturbing the gel bed, the postmitochondrial supernatant was carefully added to the column and the outflowing eluent was discarded. The column walls were rinsed gently with 1 ml of buffer and the eluent discarded. The microsomal proteins were then eluted with 1.25 ml of buffer, collected in a cryovial and stored in liquid nitrogen until analyzed.

EROD Assays: EROD assays were carried out simultaneously in 48-well plates following a modification of the method of Kennedy and Jones (1994). The assay conditions were optimized for avian microsomes. The reaction mixture consisted of 115 μ l (sample wells) and 165 μ l (blank wells) of 0.05 M sodium phosphate buffer pH 8.0, and in each well, 20 μ l of microsomes and 50 μ l of 7-ethoxyresorufin prepared in buffer from a stock solution of 876 μ M in methanol. The final substrate concentration in each well was 1.5 μ M. The plate was pre-incubated at 37°C in a block heater for 5 min and the reaction was started by the addition of 50 μ l of freshly prepared NADPH (0.2 mM in the reaction mixture) to the sample wells (not added to blank wells). The reaction was allowed to proceed for 10 min after which 100 μ l of cold acetonitrile containing fluorecamine at a concentration of 600 $\mu\text{g/ml}$ was added. After 15 min (to allow maximal and stable

fluorescence), plates were scanned with a fluorescence plate reader for resorufin at 530 nm excitation (25 nm bandwidth) and 590 nm emission wavelengths (35 nm bandwidth). Five concentrations of resorufin (from 0 to 358.4 nM) standards were prepared on each plate. Fluorescence data were imported into QPRO (version 5.0, Borland) for curve fitting.

Liver Vitamin A: 0.5 g liver samples were extracted and analyzed for retinol and retinol palmitate by non-aqueous reverse phase HPLC as described in Honour *et al.* (1995). Retinol acetate was used as the internal standard and the retinoids were detected with an ultraviolet-visible spectrophotometric detector set at a wavelength of 326 nm.

Liver Porphyrins: A 100 mg sample of liver was extracted and analyzed for porphyrins according to the method of Kennedy and James (1993), with the following modifications. The mobile phase composition went through a linear gradient from 45% methanol/ 55% 0.1 M sodium phosphate buffer (pH 3.5), to 95% methanol/ 5% 0.1 M sodium phosphate buffer pH 4.2 in 3 minutes at a flow rate of 2 ml/min. These conditions were maintained for 2 min after which the mobile phase was returned to its original composition within 2 min and kept under these conditions for an additional 2 min.

5.3.4 Histopathological analyses

After cervical dislocation, kidneys and liver (right and left lobes) were weighed on a electronic balance. Samples of brain, cerebellum, oesophagus, gastro-intestinal tract, trachea, liver, pancreas, kidneys, adrenal glands, gonads,

urophygial gland, lungs and air sacs were collected and fixed in 10% formalin. The tissues were imbedded in paraffin, and 5 to 7 μm sections were stained with hematoxyline-phloxine-safran. Some liver sections were stained with periodic acid Schiff technique for glycogen and with Oil red O for lipid visualization. Tissues were examined under light microscopy to detect any histopathological changes. Abnormal findings were graded relatively using a system of 1 (mild), 2 (moderate), and 3 (severe).

5.3.5 Statistical analyses

Comparisons of data between control and either of the two treatment groups were performed using Student t-tests. No statistical comparisons were done between treatment groups due to differences in zebra mussel diets and length of feeding trials. Pearson coefficients and probabilities were calculated with SYSTAT (version 5.03 for Windows, 1992) to determine correlations between contaminant burden and biomarker values (all groups included). To examine possible correlations between the histopathological variables and different contaminants, we used Spearman's r_s on SYSTAT. All significance levels were set at $p \leq 0.05$.

Biomagnification factors (BMFs) were calculated as follows:

$\text{BMF} = [\text{pred}] / [\text{prey}]$ where $[\text{pred}]$ is the xenobiotic concentration in lesser scaup liver and $[\text{prey}]$, the xenobiotic concentration in zebra mussel.

Liver/body mass ratio was calculated by dividing the mass of the liver by the body mass of the whole bird.

5.4 Results

5.4.1 Organic contaminant profiles

The organic contaminant profiles of the three different diets showed a concentration gradient. The commercial duck chow had non-detectable to low OC and PCB residues compared to those in zebra mussels (Table 5.1). The zebra mussels collected in the St. Lawrence River and Lake Erie had 1880 and 2511 μg total PCBs/kg lipid, respectively. PCB congeners 138, 149, 153, 182/187 and 180 composed 42% of the total PCBs in Lake Erie and 22% of that of St. Lawrence River mussels. Only congener 77 of the planar PCB congeners was found in any appreciable concentration in the zebra mussels. The ducks received approximately 565 μg total PCBs/day/duck (St-Lawrence zebra mussels) or 1171 μg total PCBs/day/duck (Lake Erie zebra mussels).

The organochlorine profile in scaup liver reflected the contaminant profile of the diet (Table 5.1). Total PCBs concentration in scaup liver were 57, 485, and 1600 $\mu\text{g}/\text{kg}$ lipid for the control, St. Lawrence River and Lake Erie groups, respectively. Di-ortho-PCB congeners 138, 153, 182/187 and 180 made up 41% and 27% of total PCBs in the liver of scaup fed zebra mussels from Lake Erie and the St. Lawrence River, respectively. DDE, a metabolite of DDT, was found in considerable amounts in both groups fed with zebra mussels.

Biomagnification factors for total PCBs in the St. Lawrence River and Lake Erie scaup were 0.5 and 1.6, respectively. However, if we look at the most important individual PCB congeners (138, 153, 180, 182-187), BMF values were between 2 and 3. Only DDE (3.1) and OCS (9.7) had BMF values above 3.

5.4.2 Porphyrin profiles

Lesser scaups fed the Lake Erie zebra mussels had a significantly lower concentration of uroporphyrin than controls ($t=3.7$, $df=14$, $p=0.002$) (Table 5.2). Uroporphyrin ($t=2.1$, $df=15$, $p=0.05$) and protoporphyrin ($t=2.3$, $df=15$, $p=0.04$) concentrations in lesser scaups from the St. Lawrence group were lower than controls. In the case of intermediate porphyrins (hepta-, hexa- and penta-porphyrins), 79% of the results were below the limit of detection which did not permit any statistical analyses. Only uroporphyrin and coproporphyrin were significantly correlated with one another and only in the Lake Erie group ($r = 0.761$; $p = 0.03$).

5.4.3 Ethoxyresorufin-*o*-deethylase (EROD)

No significant induction of EROD activity was detected between exposed and control groups (Control: 19.6; Lake Erie group: 20.9; and St. Lawrence River group: 26.5 pmole/min/mg protein). Although statistically not significant, a 35% increase in the average EROD activity was observed in the St. Lawrence group compared to the controls.

Table 5. 1 Mean concentrations ($\mu\text{g/kg}$ wet weight) for organochlorines and PCB congeners in diets used in feeding trials and lesser scaup liver.

	Zebra mussel			Lesser scaup Liver		
	St. Lawrence	Lake Erie	Duck chow	St. Lawrence	Lake Erie	Contol
Percent lipid	1.75	1.48	3.13	4.01	4.16	3.63
1245-TCB	N.D.	N.D.	N.D.	N.D.	0.09	N.D.
1234-TCB	N.D.	N.D.	N.D.	N.D.	0.16	N.D.
QCB	N.D.	N.D.	N.D.	0.06	0.09	N.D.
HCB	0.20	0.17	N.D.	0.13	0.40	0.05
OCS	N.D.	0.39	N.D.	0.13	3.77	N.D.
Nonachlor	0.58	0.27	N.D.	0.13	0.32	N.D.
HC Epoxide	N.D.	N.D.	N.D.	0.23	0.75	N.D.
oxy- Chlordane	N.D.	N.D.	N.D.	0.20	0.46	0.05
DDD	N.D.	N.D.	N.D.	0.49	0.36	N.D.
DDE	3.38	1.57	N.D.	3.32	4.85	0.54
DDT	N.D.	N.D.	N.D.	0.18	0.07	N.D.
Mirex	0.34	N.D.	N.D.	0.31	0.12	N.D.
Dieldrin	N.D.	N.D.	N.D.	0.57	1.85	N.D.
<u>Trichlorobiphenyls</u>						
#28	0.47	N.D.	N.D.	0.06	0.18	N.D.
#31	N.D.	N.D.	N.D.	1.22	0.65	N.D.
<u>Tetrachlorobiphenyls</u>						
#42	0.64	0.16	N.D.	N.D.	0.15	N.D.
#44	1.87	0.58	0.10	0.23	0.60	N.D.
#49	0.91	0.27	N.D.	0.05	N.D.	N.D.
#52	1.69	0.65	0.15	0.09	0.12	N.D.
#60	1.31	0.38	N.D.	0.17	0.25	N.D.
#64	0.25	0.05	N.D.	N.D.	N.D.	N.D.
#66-95	2.06	1.33	0.08	0.48	0.92	N.D.
#70	1.53	0.57	N.D.	0.08	0.08	N.D.
#74	1.04	0.24	N.D.	0.43	0.50	N.D.
#77	1.06	0.31	N.D.	N.D.	N.D.	N.D.
<u>Pentachlorobiphenyls</u>						
#87	1.15	0.76	0.08	0.20	0.50	N.D.
#97	0.57	0.40	N.D.	N.D.	N.D.	N.D.
#99	1.05	0.75	N.D.	0.65	1.50	N.D.
#101	2.07	1.82	N.D.	0.23	0.69	N.D.
#105	0.58	0.88	N.D.	0.31	0.60	N.D.
#110	1.61	1.51	0.07	0.11	0.25	N.D.
#118	1.45	1.39	N.D.	1.31	2.40	0.07

Hexachlorobiphenyls

#129	0.16	0.51	N.D.	0.09	0.38	N.D.
#138	1.80	3.96	0.11	1.78	8.68	0.17
#141	0.34	0.72	N.D.	0.04	0.24	N.D.
#146	0.38	0.62	N.D.	0.28	1.47	N.D.
#149	1.58	2.79	N.D.	0.11	1.39	N.D.
#151	0.83	1.10	N.D.	0.06	0.36	N.D.
#153	1.75	3.65	0.11	1.60	7.30	0.11
#158	0.14	0.22	N.D.	0.05	0.29	N.D.

Heptachlorobiphenyls

#170-190	0.52	1.34	N.D.	0.56	2.78	N.D.
#171	0.20	0.48	N.D.	0.17	0.77	N.D.
#172	0.05	0.16	N.D.	0.06	0.34	N.D.
#174	0.47	1.29	N.D.	0.09	0.70	N.D.
#180	0.82	2.73	N.D.	1.26	5.92	0.06
#182-187	0.99	2.16	N.D.	1.01	4.57	0.05
#183	0.33	0.97	N.D.	0.37	1.79	N.D.
#185	N.D.	0.11	N.D.	N.D.	0.05	N.D.

Octachlorobiphenyls

#194	0.22	0.45	N.D.	0.36	0.89	N.D.
#195	0.15	0.28	N.D.	0.18	0.52	N.D.
#200	N.D.	0.24	N.D.	0.09	0.46	N.D.
#201	0.45	0.97	N.D.	0.53	2.01	N.D.
#203	0.26	0.62	N.D.	0.37	1.24	N.D.

Nonachlorobiphenyls

#206	0.16	0.24	N.D.	0.24	0.49	N.D.
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Total PCBs	32.91	37.66	0.70	14.92	52.03	0.46
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N.D. Not detectable

Table 5. 2. Concentrations (pmol/gram weight wet) of various porphyrins in the livers of lesser scaup fed a control diet and zebra mussels from the St. Lawrence River or Lake Erie.

	Uro porphyrin ^a	Hepta porphyrin	Hexa porphyrin	Penta porphyrin	Copro porphyrin	Proto porphyrin
Control	18.00±3.46	N.D. ^b	8.67±4.00	13.67±6.66	179.63±286.42	181.13±39.01
St. Lawrence	12.56±6.30*	N.D.	10.50±3.11	8.50±3.54	88.11±104.28	142.00±32.12*
Lake Erie	10.25±4.77** ^a	N.D.	10.00±6.00	N.D	107.75±191.62	142.13±48.62

^aSignificantly different from control at * p < 0.05; ** p < 0.005.

^b Not detectable < 4 pmole/g of liver.

5.4.4 Vitamin A concentrations

There was a significant decrease in the liver retinol concentration in both the St. Lawrence group ($t = 4.12$; $df = 16$; $p = 0.001$) and the Lake Erie group ($t = 3.47$, $df = 17$, $p = 0.01$) compared to controls (Fig. 5.1a). For retinyl palmitate, the storage form of vitamin A, there was also a significant decrease ($t = 3.45$, $df = 16$, $p = 0.01$) in the St. Lawrence River but not in the Lake Erie group ($t = 1.67$; $df = 17$; $p = 0.11$) compared to the control (Fig. 5.1b). The retinol to retinyl palmitate ratio was also significantly different between the control group and both the St-Lawrence River ($t = 2.40$; $df = 16$; $p = 0.05$) and Lake Erie ($t = 2.59$; $df = 17$; $p = 0.05$) groups.

A negative correlation was observed between liver mass and both retinol ($r = -0.673$; $p < 0.001$) and retinyl palmitate ($r = -0.513$; $p = 0.005$) concentrations.

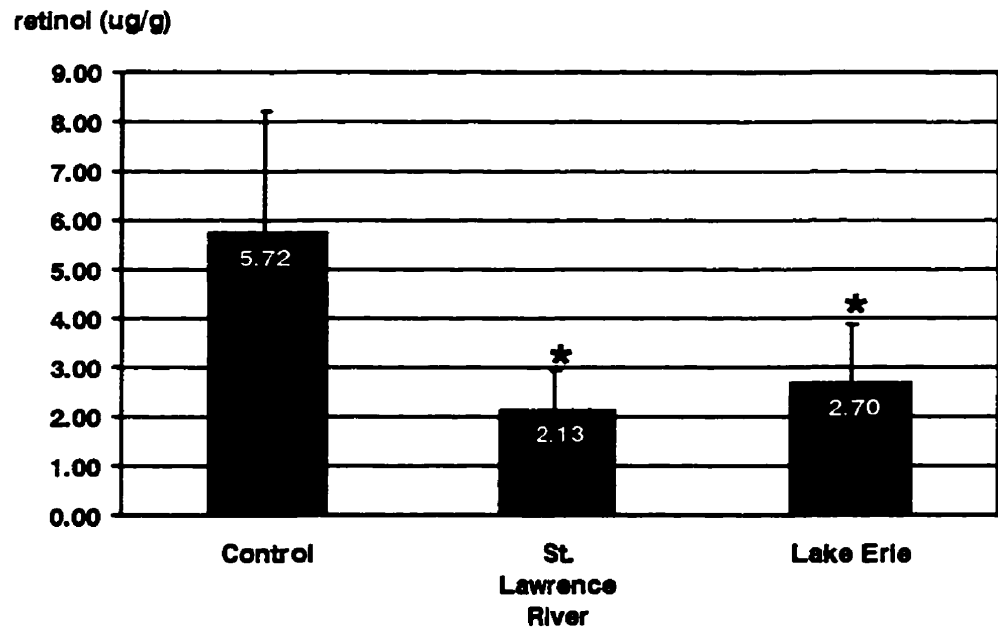
5.4.5 Relative liver mass and histopathologic results

The liver/body mass ratio of both St. Lawrence group ($t = 9.80$; $df = 17$; $p = 0.001$) and the Lake Erie group ($t = 12.69$; $df = 17$; $p = 0.001$) were significantly greater than the control (Fig. 5.2).

The liver/body mass ratios were strongly correlated with total PCBs ($r = 0.648$; $p = 0.001$).

Extensive glycogen deposition, suggestive of severe glycogen overload was observed in the livers of 9 of 10 individuals from the Lake Erie group. One individual from the Lake Erie group and one from the St. Lawrence River group had moderate glycogen overload. In these birds, the hepatocytes were

A



B

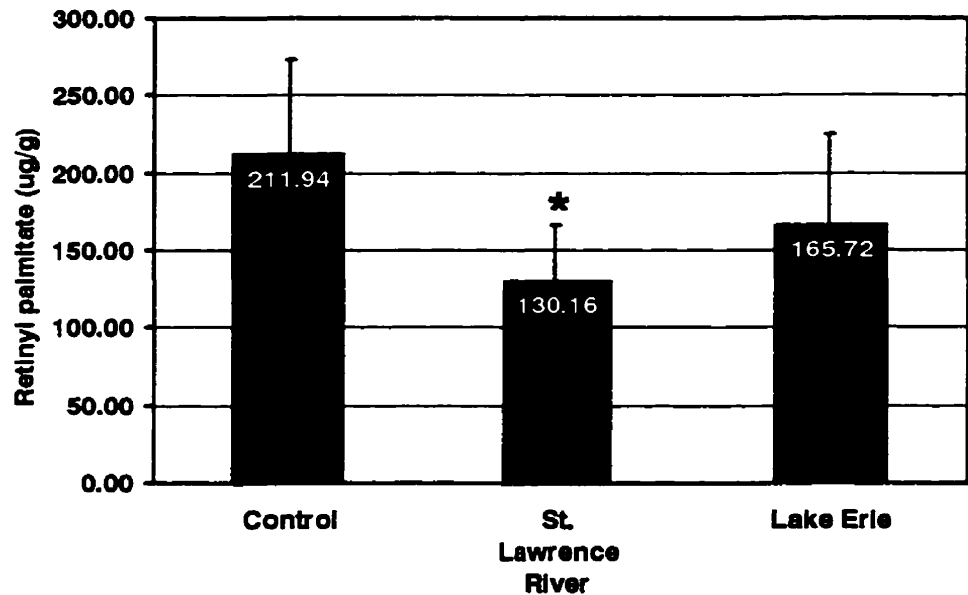


Figure 5.1. Hepatic retinol (A) and retinyl palmitate (B) concentrations of lesser scaup fed a control diet and zebra mussels from the St. Lawrence River or Lake Erie. Asterix (*) indicates a significant difference ($p < 0.05$) compared to control values (Student t test). White numbers in columns are average values.

smaller than the control group and the cytoplasm was moderately to severely vacuolized with glycogen (Fig. 5.3c). In 5 individuals of the Lake Erie group, hepatic hemorrhages were also observed. The severity of glycogen overload was strongly correlated with total PCBs ($r = 0.811$; $p < 0.001$).

The hepatocytes of the St. Lawrence group were infiltrated with fat. Of 9 individuals, 3 had mild, 5 had moderate, and 1 had severe fat infiltration. Two mild cases were also observed in the control group ($n=9$). The cytoplasm of the affected hepatocytes contained 1 to 4 vacuoles of lipids, usually tightly packed together (Fig. 5.3b). Only mirex showed a positive correlation ($r = 0.645$; $p < 0.01$) with lipid accumulation in hepatocytes in the St. Lawrence River group.

No histopathological changes were observed in any other organs examined. No parasites were detected in feces of the scaup at any time in the study.

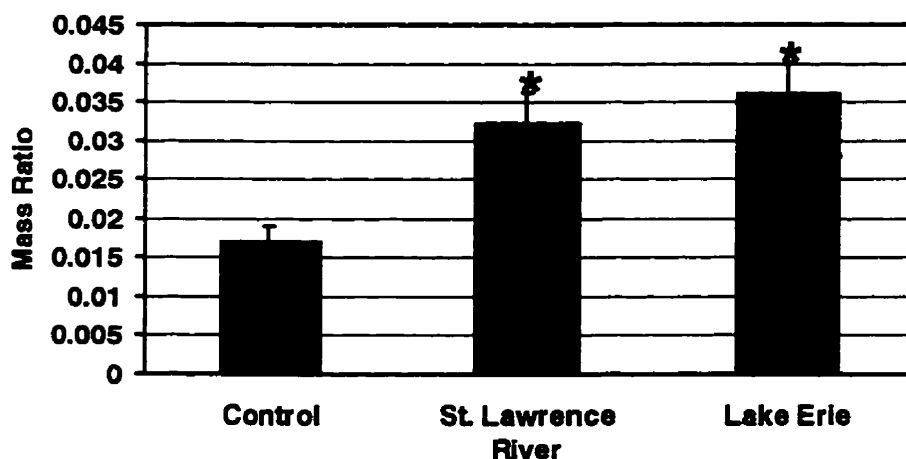
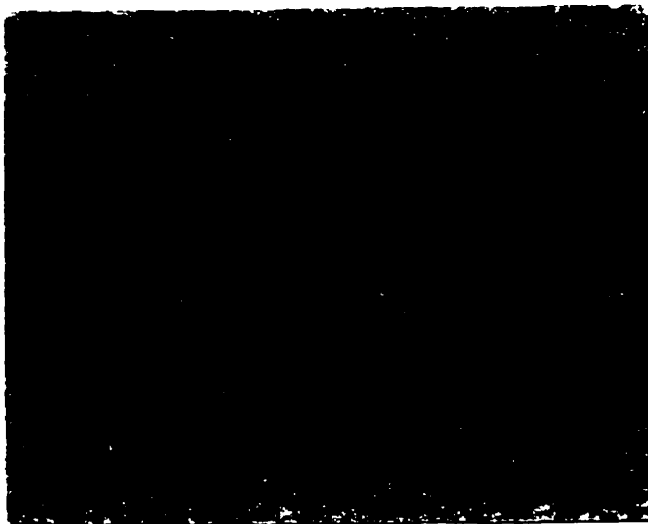
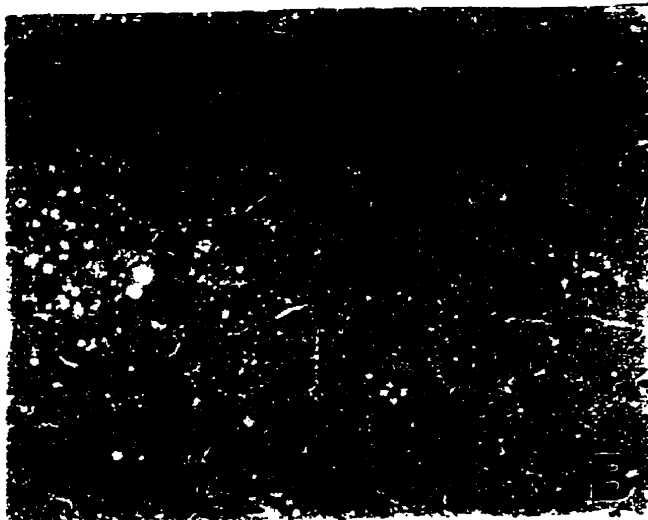


Figure 5.2. Lesser scaup liver:body mass ratio. Asterix (*) indicates a significant difference ($p < 0.05$) compared to control values (Student t test).

Figure 5.3. Histological section of liver stained with hematoxylin-phloxine-safran. A) Lesser scaup fed control diet. Normal hepatocyte with uniform cytoplasmic volume B) Lesser scaup fed St. Lawrence River zebra mussels. Presence of numerous intracytoplasmic lipid vacuoles. C) Lesser scaup fed Lake Erie zebra mussels. Severe glycogen overload. The cytoplasm contains uniformly distributed glycogen vacuoles.



5.5 Discussion

5.5.1 Organic contaminant profiles

The total PCB concentration in lesser scaup livers of 14.9 µg/kg wet weight for the St. Lawrence River group and 52.0 µg/kg wet weight for Lake Erie group are similar to those found in other studies with wild waterfowl species from moderately contaminated waters. Mazak (1995) found livers of bufflehead (*Bucephala albeola*), greater scaup and lesser scaup from the western basin of Lake Erie averaged 26.1, 6.6, and 8.2 µg/kg total PCBs wet weight, respectively. Kim and co-workers (1984) reported 75 µg/kg wet weight total PCBs in bufflehead livers from Statewide, New York.

The percentages of total PCBs retained in the liver of scaup at the end of the feeding trials were 0.6% and 1.1% for the Lake Erie and St-Lawrence River groups, respectively. These retentions of PCBs by scaup are comparable to those observed in other studies of subchronic laboratory exposure of animals to PCBs. Rats exposed to TCDD, 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) and 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) had liver retention of approximately 5, 0.5, 1%, respectively (Van der Kolk et al., 1992; Van Birgelen et al., 1994).

Biomagnification of total PCBs was observed only in the Lake Erie lesser scaups (BMF=1.6). BMF values for highly chlorinated and di-ortho congeners are expected since they are persistent (high log *k_{ow}*-constant octanol-water) xenobiotics and more difficult to metabolize by organisms (Wolff and Schecter,

1991; Brieger and Hunter, 1993). Small BMF values (1.5) were only detected in the St-Lawrence River group for congeners 180 and 206. This is probably due to different and lower log Kow profiles of OC and PCB congeners in the St. Lawrence River mussels. Mazak (1995) found most of the BMF values of wild lesser scaup feeding on zebra mussels in western Lake Erie were less than 4 (0-8).

5.5.2 Porphyrin profiles

High variability was observed in coproporphyrin concentration. The cause of this variation is not known but the phenomena was also reported in wild herring gulls exposed to several contaminants in the Great Lakes (Kennedy, 1988; Kennedy and Fox, 1990) and in quail exposed to PCBs (Leonzio *et al.*, 1996).

Despite the great variability, there was a significantly lower level of uroporphyrin and protoporphyrin in the treatment groups compared to the control group. There was no significant difference in the uroporphyrin and protoporphyrin concentrations when results were expressed on a total liver basis (instead of g of liver), which suggests that a dilution concentration effect due to the liver hypertrophy in both contaminated groups could explain the lower values for uroporphyrin and protoporphyrin. Similar results were obtained with rats fed 2,2',3',4,4',5,5'-heptachlorobiphenyl and quail fed low concentrations of PCBs (Koss *et al.*, 1993; Leonzio *et al.*, 1996).

Most research on organic xenobiotic-induced porphyria in birds was done under controlled laboratory conditions using acute doses (Carpenter *et al.*, 1984; Miranda *et al.*, 1987, 1992; Rattner, 1989; Leonzio *et al.*, 1996). A few studies have assessed the utility and applicability of porphyrin profiles as biomarkers under natural conditions for wild avian species (Fox *et al.*, 1988; Kennedy and Fox, 1990; Akins *et al.*, 1993). Those field studies and our study failed to demonstrate any significant correlation between the porphyrinogenic response and PCB or the organochlorine concentrations.

5.5.3 Ethoxyresorufin-~~o~~-deethylase (EROD)

The EROD activity in wild lesser scaups and buffleheads (n=5) shot in October 1993 in the western basin of Lake Erie were similar to those found in this study (C. Tessier, unpubl. data). EROD activity below 30 pmole/min/mg protein is considered normal and basal in herring gulls (G. Fox, pers. comm.).

A likely explanation for the lack of response of EROD resides in its inability to be induced by di-ortho PCBs. The main PCB congeners we encountered were di-ortho-substituted PCBs, which, like phenobarbital, can induce cytochrome P450B1/2. EROD, which belongs to the P450A1/2 family, is more inducible by non-ortho coplanar PCBs (Murk *et al.*, 1994) which represent less than 1% of total PCBs analysed. EROD is also inducible but to a lesser extent by mono-ortho congeners. Therefore, the lack of induction may be due to our choice of the

biomarker. Pentoxoresorufin-o-dealkylase (PROD), which belongs to the P4502A family, could be more suitable.

5.5.4 Vitamin A concentrations

The reduction in both retinol and retinyl palmitate in groups fed with zebra mussels may be attributed to a combination of both the diet and contaminant burden. From the nutrient analyses of zebra mussels, there was a small quantity of vitamin A available in the mussel (10 IU/100g) whereas minimal duck requirements are 200 IU/100g/day (Thompson, 1976). The zebra mussel vitamin A content is low in relation to levels found in other mollusks, clam (559IU/100g) and oyster (1641 IU/100g) species (Secor et al., 1993). Therefore, it is possible that the zebra mussel vitamin A concentration is not sufficient to meet the lesser scaup basic requirements.

Furthermore, polychlorinated biphenyls, polyhalogenated dibenzo-*p*-dioxins and dibenzofurans affect the metabolism of retinoids (Thunberg *et al.*, 1979; Spear *et al.*, 1986, 1989,1990; Mercier *et al.*, 1990). Spear and co-workers (1986) reported that a relatively low dose of the PCB 3,3',4,4'-tetrachlorobiphenyl decreased the concentration of retinol in the liver of doves (*Streptopelia risoria*). Herring gull livers from PCB contaminated colonies in the Great Lakes contained lower retinyl palmitate and retinol concentrations when compared to colonies living in low-pollution areas (Spear *et al.*, 1990).

Vitamin A plays an important role in the general homeostasis of the organism. Deficiencies resulting from nutritional or/and contaminant sources may have deleterious impacts on the health of the lesser scaup, including an array of physiological changes affecting secondary sexual characteristics, testes weight, spermatogenesis, egg laying, egg size, embryo survival, incubation time and hatchability. They can also cause deformities of bone and cartilage (Thompson, 1976; Spear *et al.*, 1989; Fox, 1993). Furthermore, increases in susceptibility to infectious diseases due to an altered immune response were linked to vitamin A deficiency (Friedman and Sklan, 1989; Brouwer *et al.*, 1989). Deficient vitamin A results in loss of integrity of epithelia and mucus membranes and allows a port of entry for pathogens (Wobeser and Kost, 1992). We showed that a decrease in the nonspecific immunity of lesser scaup (Chapter 4) was correlated with an increase of the incidence of bacterial foot erosion (pododermatitis) which may result indirectly from low vitamin A concentrations. More likely, immunosuppression and vitamin A deficiency were responsible for this opportunistic infection.

5.5.5 Relative liver mass and histopathologic results

Liver enlargement was observed in birds exposed to a variety of environmental pollutants (Stubblefield *et al.* 1995) and in mammals fed Aroclor 1254 (Street and Sharma, 1975; Thomas and Hinsdill, 1978). However, these enlargements are normally associated with increased protein synthesis and an augmentation in the activity of certain enzymes such as the MFO. In the present

study, the increased liver weight was highly correlated with total OC and PCB concentrations, suggesting compensatory mechanisms to cope with the increased metabolic demands for xenobiotic metabolism and elimination (Gorsline *et al.*, 1981; Stubblefield *et al.*, 1995). Furthermore, hepatic accumulation of lipid, and glycogen overload in the livers of St. Lawrence River and Lake Erie group, respectively, may also explain these increases in liver mass.

The second morphological change observed was fatty infiltration in 90% of the livers from St. Lawrence River group. This may have been triggered by nutritional stress, protein deficiencies or xenobiotics such as PCBs (Robert *et al.*, 1978; Cheville, 1983; Kelly, 1991; Cotran *et al.*, 1994). However, the lesions seen were not severe enough to alter hepatic function and would likely be reversible when the primary cause was removed. The chronic low-level exposure to the mixture of toxicants and/or dietary deficiencies may explain these results. Lake sturgeon (*Acipenser fulvescens*) and northern pike (*Esox lucius*) captured in contaminated sections of St. Lawrence River also showed hepatic fat accumulation (Audet, 1991; Rousseau *et al.*, 1995).

Glycogen overload was mainly observed in the Lake Erie group. To our knowledge, this non-specific condition has not been identified before in birds. In mammals, it is associated with excessive amounts of glucose entering the hepatic cells or a reduction/inhibition of the glycogenolysis pathway (Cheville, 1983.). The differences in the diet composition between the three groups and xenobiotics in zebra mussels may explain this pathology (Kelly, 1991). Glycogen

metabolic abnormalities have been observed in organisms under contaminant stress (Andersson *et al.*, 1987). Perch (*Perca flavescens*) caught in contaminated waters have accumulated liver glycogen (Andersson *et al.*, 1988; Hontela *et al.*, 1995). Carbohydrate metabolism is influenced by thyroid hormones interacting with corticosteroids. It was demonstrated that perch exposed to organic and metallic contamination exhibited decreased levels of cortisol and thyroxine in their blood, which may be responsible for the inhibition or decrease in the enzymatic conversion of glycogen to glucose (Hontela *et al.*, 1995). Migration stress and colder temperatures combined with the decrease of photoperiod could also trigger an accumulation of hepatic glycogen (Hochachka and Hayes, 1962; Bidwell and Heath, 1993).

The hepatic hemorrhages observed in 50% of lesser scaup from Lake Erie are probably secondary to the increased friability of the liver caused by the glycogen overload (S. Laird, pers. comm.).

5.5.6 Evaluation of biomarkers

Our failure to detect significant differences among groups for EROD activity and porphyrin profiles may stem from the natural differences in the response of individuals within the same population to the same contaminant exposure. Individual variations may be affected by genetic variability and natural fitness (Depledge *et al.*, 1993). Other variables (e.g. the mixture of contaminants) may also contribute to the failure of these biomarkers to show similar responses in laboratory and in field situations. At present, the sensitivity of these biomarkers

makes them most reliable for monitoring the effects of high contaminant doses and/or long exposure time (Leonzio *et al.*, 1996). With chronic, low contaminant exposure, these biomarkers are not as reliable for exposure monitoring in waterfowl.

Significant differences in histopathology, relative liver mass and hepatic vitamin A concentrations indicate that they may be promising field biomarkers of the deleterious impacts associated with consumption of zebra mussels. Even though we cannot differentiate the effects due to the nutritional value of zebra mussels themselves from those associated with contaminant burden, these biomarkers were sensitive enough to be used as “early” indicators to detect possible further health complications in waterfowl.

5.6 References

- Akins, J.M., M.J. Hooper, H. Miller, and J.S. Woods. 1993. Porphyrin profiles in the nestling European starling (*Sturnus vulgaris*): a potential biomarker of field contaminant exposure. *J. Toxicol. Environ. Health* 40:47-59.
- Andersson, T., B.E. Bengtsson, L. Forlin, J. Hardig, and A. Larsson. 1987. Long-term effects of bleached kraft mill effluents on carbohydrate metabolism and hepatic xenobiotic biotransformation enzymes in fish. *Ecotox. Environ. Safety* 13:53-60.
- Andersson, T., L. Forlin, J. Hardig, and A. Larsson. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. *Can. J. Fish. Aquat. Sci.* 45:1525-1536.
- Audet, C. 1991. Utilisations des communautés ichthyologiques pour mesurer l'état de santé des écosystèmes du fleuve Saint-Laurent: Analyse des indicateurs des effets sous-létaux (histologie du foie et des branchies). Technical Report. Pêches et Océans, Ottawa, Ontario, Canada.
- Bidwell, J.R., and A.G. Heath. 1993. An *in situ* study of rock bass (*Ambloplites rupestris*) physiology: effect of season and mercury contamination. *Hydrobiol.* 264:137-152.
- Brieger, G. and R.D. Hunter. 1993. Uptake and depuration of PCB 77, PCB 169, and hexachlorobenzene by zebra mussels (*Dreissena polymorpha*). *Ecotoxicol. Environ. Safety* 26:153-165.

- Brouwer, A., P.J.H. Reijnders, and J.H. Koeman. 1989. Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). *Aquatic Toxicol.* 15:99-106.
- Brunström, B. 1990. Mono-ortho-chlorinated chlorobiphenyls: toxicity and induction of 7-ethoxyresorufin *O*-deethylase (EROD) activity in chick embryos. *Arch. Toxicol.* 64:188-191.
- Cairns, J., P.V. McCormick, and B.R. Niederlehner. 1993. A proposed framework for developing indicators of ecosystem health. *Hydrobiol.* 263:1-44.
- Carpenter, H.M., D.E. Williams, M.C. Henderson, R.D. Bender, and D.R. Buhler. 1984. Hexachlorobenzene-induced porphyria in Japanese quail. *Biochem. Pharmacol.* 33:3875-3881.
- Cheville, N.F. 1983. Cell pathology. The Iowa State University Press, 2nd ed., Ames. p.681
- Chu, I., D.C. Villeneuve, A. Yagminas, P. Lecavalier, R. Poon, M. Feeley, S.W. Kennedy, R.F. Seegal, H. Håkansson, U.G. Ahlborg, and V.E. Valli. 1994. Subchronic toxicity of 3,3',4,4',5-pentachlorobiphenyl in the rat. I. Clinical, biochemical, hematological, and histopathological changes. *Fund. Appl. Toxicol.* 22:457-468.
- Cotran, R.S., V. Kumar, S.L. Robbins. 1994. Pathologic basis of disease. W.B. Saunders Company, 5th ed. Philadelphia.

- Custer, C.M., 1993. Zebra mussels as a food source for lesser scaup (*Aythya affinis*). Third International Zebra Mussel Conference, Feb. 23-26 1993, Toronto, Canada.
- De Koch, W.Ch, and C.T. Bowmer. 1993.. In: Nalepa, T.F. and D.W. Schloesser (Eds), Zebra mussels: Biology, Impacts, and control. Lewis publishers, Florida, USA. p. 503-533.
- Depledge, M.K., J.J. Amaral-Mendes, B. Daniel, R.S. Halbrook, P. Kloepper-Sams, M.N. Moore and D.B. Peakall. 1993. The conceptual basis of the biomarker approach. In: D.B. Peakall and L.R. Shugart. Biomarker. NATO ASI series, vol. H 68. Springer-Verlag, Berlin. p.15-29.
- Fox, G.A. 1993. What have biomarkers told us about the effects of contaminants on the health of fish-eating birds in the Great Lakes? The theory and a literature review. J. Great Lakes Res. 19:722-736.
- Fox, G.A. and C. Weseloh. 1987. Colonial waterbirds as bio-indicators of environmental contamination in the Great Lakes. ICBP Technical Publication no. 6:209-216.
- Fox, G.A., S.W.Kennedy, R.J. Nostrom and D.C. Wigfield. 1988. Porphyria in herring gulls: a biochemical response to chemical contamination of Great Lakes food chain. Environ. Toxicol. Chem. 7:831-839.
- French III, J.P.R. and M.T. Bur. 1993. Predation of the zebra mussel (*Dreissena polymorpha*) by freshwater drum in Western Lake Erie. In: Nalepa, T.F. and D.W. Schloesser (Eds), Zebra mussels: Biology, Impacts, and Control. Lewis Publishers, Florida, USA. p. 453-464.

- Friedman, A., and D. Sklan. 1989. Antigen-specific immune response impairment in the chick as influenced by dietary vitamin A. *J. Nutr.* 119:790-795.
- Géroudet, P. 1966. Premières conséquences ornithologiques de l'introduction de la moule zébrée *Dreissena polymorpha* dans le lac Léman. *Nos Oiseaux* 28:301-307.
- Giddings, J.M. 1986. Protecting aquatic resources: an ecologist's perspective. In: Poston, T.M., and Purdy, R. (Eds), Aquatic Toxicology and Environmental Fate, ASTM STP 921. American Society for testing and materials, Philadelphia, Pa. Pp.97-106.
- Gilbertson, M. and G.A. Fox. 1977. Pollutant-associated embryonic mortality of Great Lakes herring gulls. *Environ. Pollut.* 12:211-216.
- Gorsline, J., W.N. Holmes and J. Cronshaw. 1981. The effects of ingested petroleum on the naphthalene-metabolizing properties of liver tissue in seawater-adapted mallard ducks (*Anas platyrhynchos*). *Environ. Res.* 24:377-390.
- Hamilton, D.J. 1992. A method for reconstruction of zebra mussel (*Dreissena polymorpha*) length from shell fragments. *Can. J. Zool.* 70:2486-2490.
- Hamilton, D.J., C.D. Ankney, and R.C. Bailley. 1994. Predation of zebra mussels by diving ducks: an exclosure study. *Ecology* 75:521-531.
- Hochachka, P.W., and F.R. Hayes. 1962. The effect of temperature acclimation on pathways of glucose metabolism in the trout. *Can. J. Zool.* 40:261-270.

- Honour, S.M., S. Trudeau, S. Kennedy, and G. Wobeser. 1995. Experimental vitamin A deficiency in mallards (*Anas platyrhynchos*): lesions and tissue vitamin A levels. *J. Wildl. Dis.* 31:277-288.
- Hontela, A., P. Dumont, D. Duclos, and R. Fortin. 1995. Endocrine and metabolic dysfunction in yellow perch, *Perca flavescens*, exposed to organic contaminants and heavy metals in the St. Lawrence River. *Environ. Toxicol. Chem.* 14:725-731.
- Kelly, W.R. 1991. The liver and biliary system. In: Jubb, K.V.F., P.C. Kennedy, N. Palmer. 4th Ed. Pathology of domestic animals. Academic Press, San Diego. pp.319-406.
- Kennedy, S.W. and C.A. James. 1993. Improved method to extract and concentrate porphyrins from liver tissue for analysis by high-performance liquid chromatography. *J. Chromatog.* 619:127-132.
- Kennedy, S.W., and S.P. Jones. 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem.* 222:217-223.
- Kennedy, S.W. and G.A. Fox. 1990. Highly carboxylated porphyrins as a biomarker of polyhalogenated aromatic hydrocarbon exposure in wildlife: confirmation of their presence in Great Lakes herring gull chicks in the early 1970's and important methodological details. *Chemosphere* 21:407:415.

- Kennedy, S.W. 1988. Studies on porphyrins as an indicator of polyhalogenated aromatic hydrocarbon exposure. PhD dissertation, Carleton University, Ottawa, Canada.
- Kim, K.S., M.J. Pastel, J.S. Kim, and W.S. Stone. 1984. Levels of polychlorinated biphenyls, DDE, and mirex in waterfowl collected in New York State, 1979-1980. Arch. Environ. Contam. Toxicol. 13:373-381.
- Koss, G, E.S. Schuler, B. Arndt, J. Seidel, S. Seubert, and A. Seubert. 1986. A comparative toxicological study of pike (*Esox lucius* L.) from the River Rhine and River Lahn. Aquat Toxicol. 8:1-9.
- Lazar, R., R.C. Edwards, C.D. Metcalfe, T. Metcalfe, F.A.P.C. Gobas and G.D. Haffner. 1992. A simple, novel method for the quantitative analysis of coplanar polychlorinated biphenyls in the environment. Chemosphere 25:493-504.
- Leonzio, C., M.C. Fossi, and S. Casini. 1996. Porphyrins as biomarkers of methylmercury and PCB exposure in experimental quail. Bull Environ. Contam. Toxicol. 56:244-250.
- Lewis, L.D., M.L. Morris and M.S. Hand. 1987. Alimentation clinique des petits animaux III. Mark Morris Associates (eds), Topeka, Texas.
- Mazak, E.J.. 1995. Organic contaminants in lower Great Lakes' waterfowl in relation to diet, with particular reference to *Dreissena polymorpha*. . M.Sc Thesis, U. Windsor, Ontario, 111p.

- Mercier, M., G. Pascal, and V. Azais-Braieso. 1990. Retinyl ester hydrolase and vitamin A status in rats treated with 3,3',4,4'-tetrachlorobiphenyl. *Biochem. Biophys. Acta* 1047:70-76.
- Miranda, C.L., M.C. Henderson, J.-L. Wang, H.S. Nakaue, and D.R. Buhler. 1987. Effects of polychlorinated biphenyls on porphyrin synthesis and cytochrome P-450-dependent monooxygenases in small intestine and liver of Japanese quail. *J. Toxicol. Environ. Health* 20:27-35.
- Miranda, C.L., M.C. Henderson, J.-L. Wang, H.S. Nakaue, and D.R. Buhler. 1992. Comparative effects of the polychlorinated biphenyl mixture, Aroclor 1242, on porphyrin and xenobiotic metabolism in kidney of Japanese quail and rat. *Comp. Biochem. Physiol.* 103C:149-152.
- Murk, A., D. Moore, J. Boon, and A. Brouwer. 1994. *In vitro* metabolism of 3,3',4,4'-tetrachlorobiphenyl in relation to ethoxyresorufin-*o*-deethylase activity in liver microsomes of some wildlife species and rat. *Europ. J. Pharmacol.* 270:253-261.
- New York Sea Grant. 1996. Distribution map of zebra mussels in North America.
- Pedroli, J.C. 1981. Les relations entre la moule zébrée *Dreissena polymorpha* (Pallas) et les oiseaux aquatiques. Dissertation. Université de Neuchâtel, Neuchâtel, Switzerland.
- Prejs, A., K. Lewandowski, and A. Stanczykowska-Piotrowska, 1990. Size-selective predation by roach (*Rutilus rutilus*) on zebra mussel (*Dreissena polymorpha*): field studies. *Oecologia* 83:378-384.

- Pyykko, T. 1983. Characterization and stability of rat liver microsomes isolated by a rapid gel filtration method. *Acta Pharmacol. Toxicol.* 52:39-46
- Rattner, B.A., D.J. Hoffman and C.M. Marn. 1989. Use of mixed-function oxygenases to monitor contaminant exposure in wildlife. *Environ. Toxicol. Chem.* 8:1093-1102.
- Roberts, J.R., D.W. Rodgers, J.R. Baily, and M.A. Rorke. 1978. Polychlorinated biphenyls: Biological criteria for an assessment of their effects on environmental quality. Technical Report 16077. National Research Council of Canada, Ottawa, Ontario, Canada.
- Rousseaux, C. G., A. Branchaud, and P.A. Spear. 1995. Evaluation of liver histopathology and EROD activity in St. Lawrence lake sturgeon (*Acipenser fulvescens*) in comparison with a reference population. *Environ. Toxicol. Chem.* 14:843-849.
- Schmidt, E. 1975. Die Ergebnisse der ersten November-Entenzählung in der VR Ungarn. *Der Falke* 22:88-91.
- Scholten, M.C.T., E. Foekema, W.C. de Kock and J.M. Marquenie, 1989. Proc. 2nd European Symposium on Avian Medicine and Surgery, 8-11 March 1989, Utrecht, The Netherlands.
- Secor, C.L., E.L. Mills, J. Harshbarger, H.T. Kuntz, W.H. Gutenmann and D.J. Lisk. 1993. Bioaccumulation of toxicants, elements and nutrient composition, and soft tissue histology of zebra mussels (*Dreissena polymorpha*) from New York State Waters. *Chemosphere* 26:1559-1575.

- Snyder, F.L. 1990. Fact sheet O45, Ohio Sea Grant College Program, Columbus 4 p.
- Snyder, R.L., and J. Terry. 1986. Avian nutrition. In: M.E. Fowler (Ed.), Zoo and Wild Animal Medicine, W.B. Saunders Co. Toronto.
- Spear, P.A. D.H. Bourbonnais, D.B. Peakall and, T.W. Moon,. 1989. Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3',4,4'-tetrachlorobiphenyl. Can. J. Zool. 67:908-911.
- Spear, P.A. T.W. Moon, D.B. Peakall. 1986. Liver retinoid concentrations in natural populations of herring gull (*Larus argentatus*) contaminated by 2,3,7,8-tetrachlorodibenzo-p-dioxin and in ring doves (*Streptopelia risoria*) injected with a dioxin analogue. Can. J. Zool. 64:204-208.
- Spear, P.A., D.H. Bourbonnais, R.J. Norstrom, and T.W. Moon. 1990. Yolk retinoids (Vitamin A) in eggs of the herring gull and correlations with polychlorinated dibenzo-p-dioxins and dibenzofurans. Environ. Toxicol. Chem. 9:1053-1061.
- Stanczykowska, A. 1977. Ecology of *Dreissena polymorpha* (Pall.) (Bivalvia) in lakes. Pol. Arch. Hydrobiol. 24:461-530.
- Stanczykowska, A., P. Zyska, A. Dombrowska, H.Kot, and E. Zyska. 1990. The distribution of waterfowl in relation to mollusc populations in the man-made Lake Zegrzynskie. Hydrobiologia 191:233-240.
- Strayer, D.L., 1991. Projected distribution of zebra mussel, *Dreissena polymorpha* in North America. Can. J. Fish. Aquat. Sci. 48:1389-1395.

- Street, J.C. and R.P. Sharma. 1975. Alteration of induced cellular and humoral immune responses by pesticides and chemicals of environmental concern: Quantitative studies of immunosuppression by DDT, Aroclor 1254, Carbaryl, carbofuran, and methylparathion. *Toxicol. Appl. Pharmacol.* 32:587-602.
- Stubblefield, W.A., Hancock, G.A., W.H. Ford, and R. K. Ringer. 1995. Acute and subchronic toxicity of naturally weathered *Exxon Valdez* crude oil in mallards and ferrets. *Environ. Toxicol. Chem.* 14:1941-1950.
- Suter, W. 1982. Vergleichende Nahrungsökologie von überwinternden Tauchenten (*Bucephala*, *Aythya*) und Blabhn (*Fulica atra*) am Untersee-Ende/Hochrhein (Bodensee). *Der Ornithologische Beobachter* 79:225-24.
- Swain, M.G. , S.B. Follows, and G.S. Marks. 1983. Inhibition of uroporphyrinogen decarboxylase by 3,3',4,4'-tetrachlorobiphenyl in chick embryo liver cell culture. *Can. J. Physiol. Pharmacol.* 61:105-108.
- Thomas, P.T. and R" D" Hinsdill. 1978. Effect of polychlorinated biphenyls on the immune responses of rhesus monkeys and mice. *Toxicol. Appl. Pharmacol.* 44:41-51.
- Thompson, N.J. 1976. Fat-soluble vitamins. *Comp. Anim. Nutr.* 1:99-135.
- Thunberg, T. U.G. Ahlborg, and H. Johnson. 1979. Vitamin A (retinol) status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch. Toxicol.* 42:265-274.
- Van Birgelen, A.P.J.M., J. Van der Kolk, K.M. Fase, I. Bol, H. Poiger, M. Van den Berg, and Q. Brouwer. 1994. Toxic potency of 2,3,3',4,4',5-

- hexachlorobiphenyl relative to and in combination with 2,3,7,8-tetrachlorodibenzo-p-dioxin in a subchronic feeding study in the rat. *Toxicol. Appl. Pharmacol.* 126:202-213.
- Van der Kolk, J., A.P.J.M. Van Birgelen, H. Poiger, and C. Schlatter. 1992. Interaction of 2,2',4,4,4',5,5'-hexachlorobiphenyl and 2,3,7,8-tetrachlorodibenzo-p-dioxin in a subchronic feeding study in the rat. *Chemosphere* 25:2023-2027.
- Wallack, J.D. and W.J. Boever. 1983. Diseases of exotic animals: medical and surgical management. W.S. Saunders Co. (eds), Toronto.
- Wolff, M.S. and A. Schecter. 1991. Accidental exposure of children to polychlorinated biphenyls. *Arch. Environ. Contam. Toxicol.* 20:449-453.
- Wobeser, G., and W. Kost. 1992. Starvation, staphylococcosis, and vitamin A deficiency among mallards overwintering in Saskatchewan. *J. Wildl. Dis.* 28:215-222.
- Wormington, A. and J.H. Leach. 1992. Concentrations of migrant diving ducks at Point Pelee National Park, Ontario, in response to invasion of zebra mussels, *Dreissena polymorpha*. *Can. Field-Nat.* 106:376-380.

CONNECTIVE STATEMENT LINKING CHAPTERS 5 AND 6

The previous chapter indicated that xenobiotics may play a important role in health impairment of the ducks. In the next chapter, we evaluated the potential of zebra mussel as a sentinel species for metallic contamination. We demonstrated that cadmium is not regulated by the zebra mussel even at trace exposure concentrations and that metallothionein, a metal binding protein biomarker, was not induced as generally observed in organisms exposed to high concentrations of heavy metals.

CHAPTER 6

DETERMINATION OF CADMIUM-METALLOTHIONEINS IN ZEBRA MUSSELS EXPOSED TO SUBCHRONIC CONCENTRATIONS OF Cd^{2+}

6.1 Abstract

Metallothioneins have been detected and investigated in zebra mussels (*Dreissena polymorpha*) using high performance liquid chromatography (size exclusion) coupled with microatomization-AAS or inductively coupled plasma mass spectrometry. The mussels were exposed to 0.2, 2, and 20 $\mu\text{g/liter}$ Cd^{2+} (as CdCl_2) for 1 month under controlled temperature and dietary conditions. Elevated (relative to control) concentrations of tissue Cd^{2+} were detected in all specimens exposed to 2 $\mu\text{g Cd/liter}$ and more than 50 percent of the specimens exposed to 0.2 $\mu\text{g Cd/liter}$, demonstrating that *Dreissena* cannot regulate Cd^{2+} at trace exposure concentrations. In most specimens, at least 85% of the measured Cd^{2+} was bound to metallothioneins. After reduction and exposure to excess Cd^{2+} , the metallothionein fraction of all extracts adsorbed similar quantities of Cd^{2+} , indicating that the physiological concentration of metallothionein in the exposed specimens remained similar to the basal concentration in the control specimens. Thus, a short-term exposure to environmentally relevant concentrations of Cd^{2+} did not produce a genetic

induction of metallothionein biosynthesis as generally observed in specimens exposed to higher concentrations of d^{10} metals.

6.2 Introduction

Over the past 5 years, the Great Lakes watershed experienced massive increases in the population of an invading exotic species, the zebra mussel (*Dreissena polymorpha*). Zebra mussels, introduced into Canada via ships' ballasts, were first reported in Lake St-Clair (Hebert *et al.*, 1989). Since then, they successfully colonized the Great Lakes and their tributaries (Griffiths *et al.*, 1991, Secor *et al.*, 1993). Densities and biomasses of zebra mussel populations have grown to levels hundreds times those of native species occupying the same ecological niche. The zebra mussel has a relatively high filtering capacity (Reeders *et al.*, 1989, Hamburger *et al.*, 1990) which makes it a potential sentinel species reflecting the toxic burden of aquatic ecosystems. *Dreissena* may withstand relatively high metal concentration factors (Bias and Karbe, 1985) and appears to be a relevant species in the monitoring of heavy metal dynamics in rivers and lakes (Léglise and Crochard, 1987; Kraak *et al.*, 1991; de Kock and Bowmer, 1993). The dynamics of metal uptake by the zebra mussel are dependent on the metallic species and their concentrations. The essential elements, zinc and copper, are not accumulated by zebra mussels exposed to low concentrations (below 28 ug Zn/liter and 191 ug Cu/liter), suggesting a homeostatic regulation for these elements (Kraak *et al.*, 1993). On the other hand, the mussels accumulated Cd at water concentrations as low as 9 µg/liter, excluding the possibility of a homeostatic control. The histochemical localization of accumulated cadmium in this species has been characterized (Herwig *et al.*, 1989). The staining method allowed for the visualization of free and loosely

bound Cd^{2+} species which were detected mainly in the epithelial cells in all tissues except gametes and muscles.

One of the most studied biochemical responses of animal cells during exposure to metals is the induction of metallothioneins (Engel and Brouwer, 1989; Vallee, 1991). These metalloregulated polypeptides bind the metals that cause their induction. Consequently, they are believed to have a significant role in the homeostatic control of group I-B and II-B essential metals (Zn^{2+} , Cu^{+}) and in the sequestration of toxic heavy metals (Cd^{2+} , Hg^{2+}). These metals increase the synthesis rate of metallothionein (MT) messenger-RNA via transcriptional activation of metal-responsive factors located in the upstream region of MT genes (Dameron *et al.*, 1993; Labbé *et al.*, 1993). This genetic response is often referred to as "induction". On average, mammalian MT contains 60-62 amino acid residues of which 20 are cysteines. Criteria for positive identification of mammalian MT are the absence of aromatic amino acids as well as a highly conserved sequential order of cysteine and basic amino acids (Kagi and Schaffer, 1988). Other intrinsic properties of MT include resistance to thermocoagulation and acid precipitation, properties that are often exploited in the crude separation of these polypeptides from high-molecular-weight proteins in solution. Typically, liver or kidney MT may be obtained in milligram quantities by exposing the animal specimen to relatively high levels of transition elements with d^{10} electronic configuration.

Inducible metallothionein-like proteins have been detected and/or characterized in several molluscan species (Roseijadi *et al.*, 1989, Couillard *et*

al., 1993; Mackay *et al.*, 1993, High *et al.* 1995). Mussel MTs are generally extracted from the soft tissues of the whole animal or from dissected hepatopancreas and gill fractions. The primary structure of oyster MT differs from mammalian MT with some 8 additional amino acid residues, the absence of methionine at the N-terminal, and an elevated proportion of glycine (Mackay *et al.*, 1993). The quantity of cysteine residues (20) and the divalent metal:MT stoichiometry (between 6:1 and 7:1) were similar to those found in mammalian MT. The presence and role of MT in the accumulation of metals in zebra mussels have not been characterized.

The objectives of the present work were a) to determine if MTs are present in zebra mussel tissues; b) to determine if these MTs were induced during exposure to environmentally relevant concentrations of Cd^{2+} , and c) to determine the proportion of tissue Cd bound to MTs.

6.3 Materials and Methods

6.3.1 Exposure Protocol

Zebra mussels, 10-20 mm in length, were field sampled in the Soulange Canal (Québec) and acclimatized to the experimental conditions for a period of 1 month. The mussels were then grouped ($n = 100$) in baskets made from PTFE laboratory matting (VWR Scientific) which were suspended 20 mm from the bottom of 10 L aquaria filled to capacity with continuously aerated tap water

maintained at 12°C. This water was dechlorinated by air bubbling treatment for 24 hr, and contained less than 0.1 µg/liter Cd. Each group of mussels was fed daily with 0.36 g of microalgae (*Chlorella*, ACTA Pharmacal Co., CA) which was suspended in distilled water and treated in a high-pressure valve homogenizer (Dion *et al.*, 1994) or a ballistic homogenizer (Foss Electric, Copenhagen, Denmark) to obtain an homogeneous suspension. Three groups were exposed to 0.2, 2, and 20 µg/liter Cd²⁺ (as CdCl₂), respectively. To avoid recycling of Cd²⁺ through absorption of contaminated feces/pseudofeces and to keep the water Cd²⁺ concentration relatively constant, the water was replaced and spiked with the appropriate amount of Cd²⁺ daily. Ten mussels per group were sampled on Days 5, 8, 12, 16, 18, 24, and 28 of the experiment. The sampled mussels were replaced by an equivalent number of acclimatized specimens which were placed in a separate compartment of the PTFE basket. The specimens were immediately frozen at -80°C until analysis.

6.3.2 Analysis of total cadmium

Total Cd in whole mussel tissues was determined (n = 3) using a modification of a flow injection-microatomization-AAS method developed previously (High *et al.*, 1992). The whole mussel tissue was weighed in a 10 ml volumetric flask and suspended in 4 ml of 30 mM Tris-HCl buffer, pH 8.6, containing 1 mM EDTA using a small homogenizer probe (Tekmar, Cincinnati, OH). The probe was washed with buffer and the suspension volume was made

to 10 ml. The suspension was diluted 3.3 fold in buffer and emulsified in a high-pressure valve homogenizer (Dion *et al.*, 1994). The resulting emulsion was injected and analyzed by the microatomizer-AAS system using an automatic injector (ICI model 1610). The method of standard additions was used to calibrate the system.

6.3.3 Determination of Cd-MT Complexes

The native metal profile of MT extracted from a control specimen was also analyzed by HPLC (size exclusion)-ICP-MS, under conditions described previously (High *et al.*, 1995). Quantitative analyses of Cd-MT were obtained for specimens sampled ($n = 3$) on days 5, 8, 12, 16, and 28. Whole mussel tissue was homogenized as described above in 30 mM Tris-HCl buffer, pH 8.6, containing 1 μ M phenylmethylsulfonyl fluoride (peptidase inhibitor). A 1.5 ml of the homogenate was transferred in a 2.0 ml conical Nalgene tube which was immersed in a thermostated water bath (80°C) for 5.0 min. The samples were cooled and centrifuged at 13,000 g for 20 min. The supernatant was filtered (0.45 μ m) and analyzed by HPLC (size exclusion)-microatomization-AAS. The total Cd²⁺ binding capacity of zebra mussel MT in these samples was obtained using a Cd²⁺ saturation treatment under reducing conditions (High *et al.*, 1995). Briefly, the filtered supernatant was spiked with Cd²⁺ (to 1.9 ug Cd/mL) and mercaptoethanol (to 20 mM), incubated at 4°C for at least 10 hr, and reanalyzed by HPLC-microatomization-AAS. In a subsequent experiment, the Cd²⁺-

saturated mussel extracts were pooled and incubated at 80°C. Samples (n = 3) were taken every 5 min for 20 min and analyzed for MT-bound Cd²⁺ and free (or loosely bound) Cd²⁺.

6.4 Results

The control groups and the three exposed groups did not suffer from dose-related mortality during the period of exposure. Each group maintained comparable filtration rates, evaluated by similar rates of disappearance of the suspended *Chlorella* cells used to feed the specimens. The increases in whole tissue Cd²⁺ concentrations as a function of exposure time to 0.2, 2.0, and 20 µg Cd/liter are plotted in Figure 6.1. Specimens exposed to 0.2 µg Cd/liter contained slightly elevated tissue concentrations of Cd²⁺ relative to controls. A small time-dependent accumulation of Cd²⁺ was observed in specimens exposed to 2.0 µg Cd/liter. A more pronounced time-dependent Cd²⁺ accumulation was recorded in specimens exposed to 20 µg Cd/liter. HPLC (size exclusion)-microatomizer-AAS chromatograms including the MT-bound Cd²⁺ and free Cd²⁺ peaks are presented in Figure 6.2. Clearly, a major proportion of the chromatographed Cd²⁺ was bound to the MT fraction comprising two species which were not completely resolved under these size exclusion HPLC conditions. The amount of Cd²⁺ bound *in vivo* to MT as a function of exposure time to 0.2, 2.0, and 20 µg Cd/liter is presented in Figure 6.3 (bottom). These curves paralleled the total

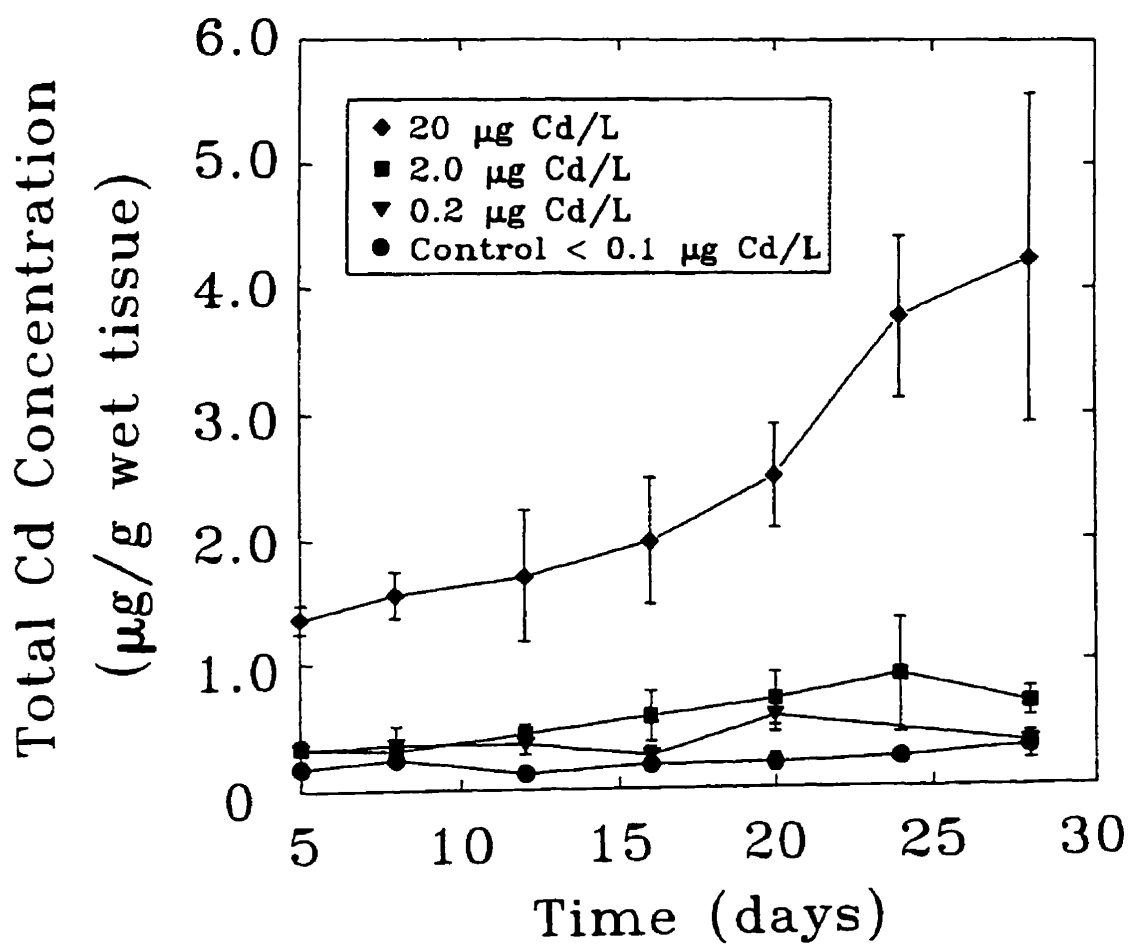


Figure 6.1. Cadmium uptake by zebra mussels exposed to various water concentrations of Cd²⁺ (as CdCl₂). Mean ± SE; n = 3.

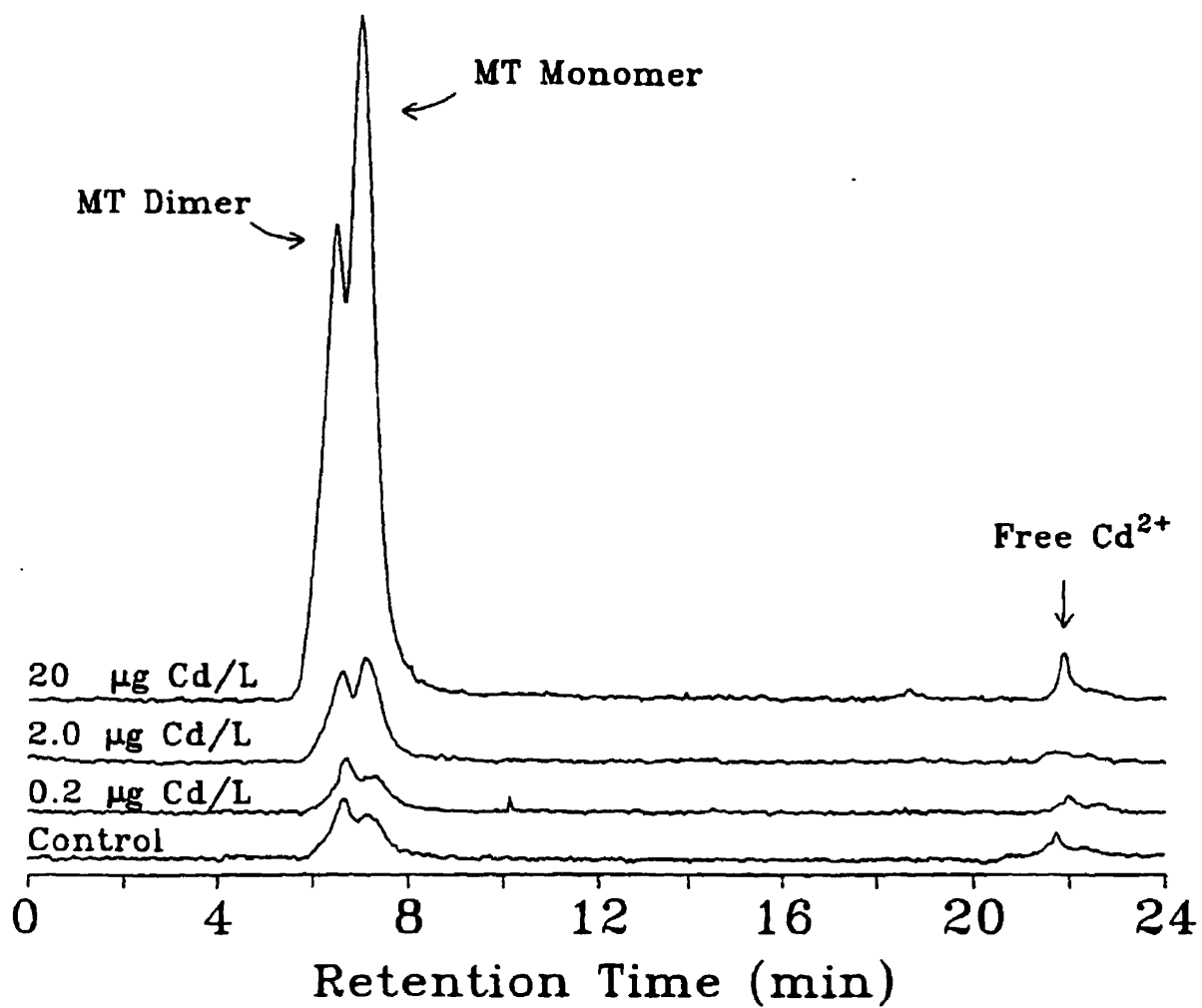


Figure 6.2. HPLC (size exclusion)-microatomization-AAS chromatograms of crude mussel extracts. The first 2 peaks are proposed to be Cd-MT dimeric and Cd-MT monomeric forms, respectively. The third peak represents free or "weakly bound" Cd²⁺.

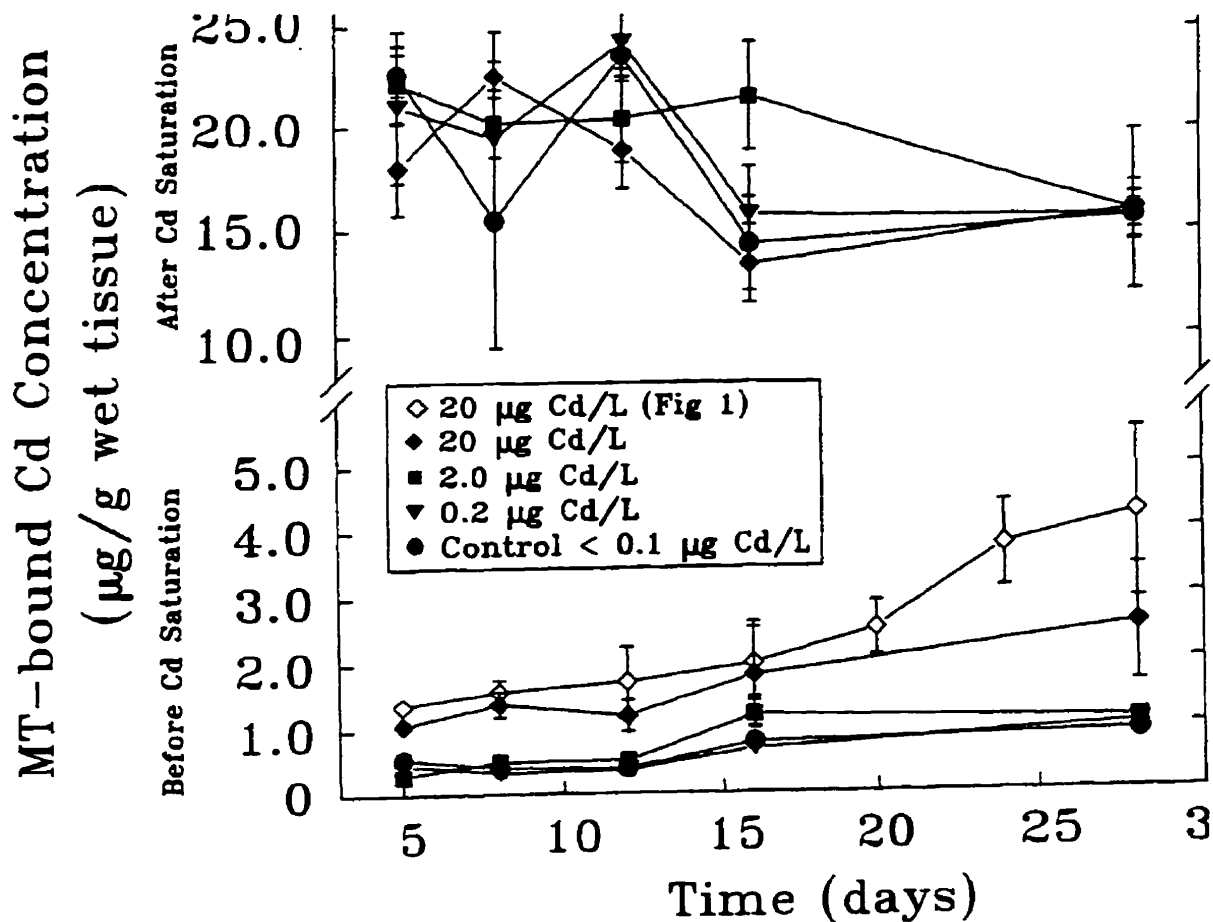


Figure 6.3. Concentration of cadmium bound to metallothioneins extracted from specimens exposed to Cd^{2+} (as CdCl_2). Mean \pm SE, $n = 3$. The total cadmium uptake in the 20 µg/liter group (Figure 6.1) was included (open diamonds) as a comparative basis.

Cd^{2+} absorption curves (Figure 6.1). In most specimens, at least 85% of the measured Cd^{2+} was bound to MT. A break in parallelism of the total Cd^{2+} and MT-bound Cd^{2+} accumulation curves of the 20 μg Cd/liter group (Figure 6.3, bottom) after 16 days of exposure suggested that the physiological MT pool was saturable *in vivo*. On the other hand, a chemical reduction/Cd-saturation treatment (Figure 6.3, top) revealed that the quantity of Cd^{2+} adsorbed *in vitro* by the MT was about 10 times higher than that adsorbed *in vivo*. The *in vitro* Cd^{2+} adsorption capacities were similar in the control and treated specimens for the duration of the experiment. This consistent adsorption capacity indicated that the total concentration of MT in the specimens of all groups remained essentially constant. Incubating the Cd-saturated samples at 80°C resulted in a migration of more Cd^{2+} cations to the MT fraction (Figure 6.4).

6.5 Discussion

In these first experiments on the physicochemical properties of MT in *Dreissena polymorpha*, experimental conditions were selected in order to obtain information on the physiological MT pool in specimens exposed to subchronic doses of Cd^{2+} which are representative of environmental conditions. The higher dose of 20 μg Cd/liter was selected as representative of the highest concentrations of Cd found in the Great Lakes Basin. Mussels exposed to contaminated waters are known to modulate their filtration rates in a dose-

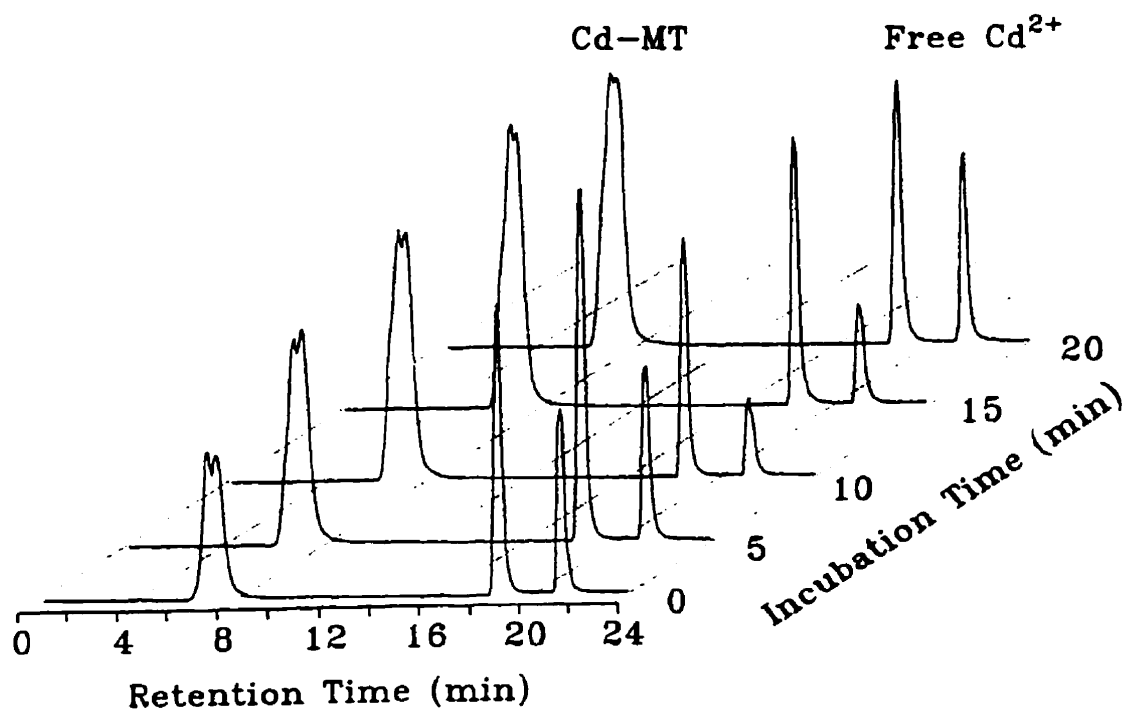


Figure 6.4. HPLC (size exclusion)-microatomization-AAS chromatograms of a crude mussel extract which was pre-saturated with Cd²⁺ and then incubated at 80°C. Peaks at *circa* 20 and 22 min represent free or weakly bound Cd²⁺.

dependent response which is used as a biomarker of acute toxicity. The minimum dose required to decrease significantly the filtration rate of a group of 25 zebra mussels exposed for 48 hr has been evaluated at 175 $\mu\text{g Cd/liter}$ (Kraak *et al.*, 1993). Another team determined that a dose of 45 $\mu\text{g Cd/liter}$ for 27 days did not affect the filtration rate of a group of 150 mussels (Mersch *et al.*, 1993). Thus, with no documented observable effects, the higher dose used in these 25-day exposure experiments was considered to be in the subchronic range.

The cadmium absorption curves for zebra mussels exposed to 20 $\mu\text{g Cd/liter}$ (figure 6.1) corroborated previous reports which demonstrated an accumulation of Cd^{2+} in the tissues of mussels exposed to concentrations as low as 5 $\mu\text{g Cd/liter}$ (Mersch *et al.*, 1993). Kraak *et al.* (1992) observed that *Dreissena* cannot regulate Cd^{2+} when exposed to concentrations between 9 and 399 $\mu\text{g Cd/liter}$. The low limit of detection of the flow injection microatomizer-AAS used to determine Cd^{2+} in these specimens (High *et al.*, 1992) allowed the determination of Cd^{2+} absorption curves at much lower exposure levels (Figure 6.1, 2 and 0.2 $\mu\text{g Cd/liter}$). The fact that elevated (relative to control) concentrations of tissue Cd^{2+} were detected in all specimens exposed to 2 $\mu\text{g/liter}$ and more than 50% of the specimens exposed to 0.2 $\mu\text{g/liter}$ demonstrated that *Dreissena* cannot regulate Cd^{2+} at trace exposure concentrations.

Metallothioneins have been suggested to be key elements causing the retention of cadmium in mussels (Viarengo *et al.*, 1980, Hemelraad *et al.*, 1986). Previous studies based on an *in vitro* mercury saturation assay have suggested that MT may be induced in freshwater mussels (*Anodonta grandis grandis*) chronically exposed to low concentrations of Cd^{2+} (Couillard *et al.*, 1993; Malley *et al.*, 1993). A reduction/Cd-saturation treatment coupled with HPLC-spectrometry techniques was used previously to characterize the metal profiles and redox characteristics of MT extracted from *A. grandis grandis* (High *et al.*, 1995). The same techniques were suitable for the detection and characterization of zebra mussel MT. The HPLC (size exclusion)-microatomizer-AAS profiles of zebra mussel extracts (Figure 6.2) were virtually identical to those observed previously in *A. grandis grandis*. Noteworthy was the fact that extracts from several specimens, especially those exposed to Cd^{2+} , comprised two size-exclusion chromatographic peaks. These peaks have been tentatively assigned as dimeric and monomeric forms of mussel MT which have been characterized in marine mussels by Mackay *et al.* (1993).

An important criterion for positive identification of a native MT is a very characteristic metal content. Under normal physiological conditions, the metal profile of invertebrate MT includes a high proportion of Cu, Zn, and, occasionally (environmental contamination), Cd (High *et al.*, 1995). This typical metal profile of molluscan MT was observed in a control zebra mussel extract using a simultaneous multielemental mass spectrometer (ICP-MS) which was connected at the outlet of the HPLC size-exclusion column (Figure 6.5).

Another criterion for positive identification of MT in *Dreissena* was response to an *in vitro* reduction/Cd-saturation treatment. If all thiol groups of MT cysteine residues are in the reduced state, each MT molecule can assume a tetrahedral coordination of seven divalent metal atoms (Zn^{2+} and/or Cd^{2+})

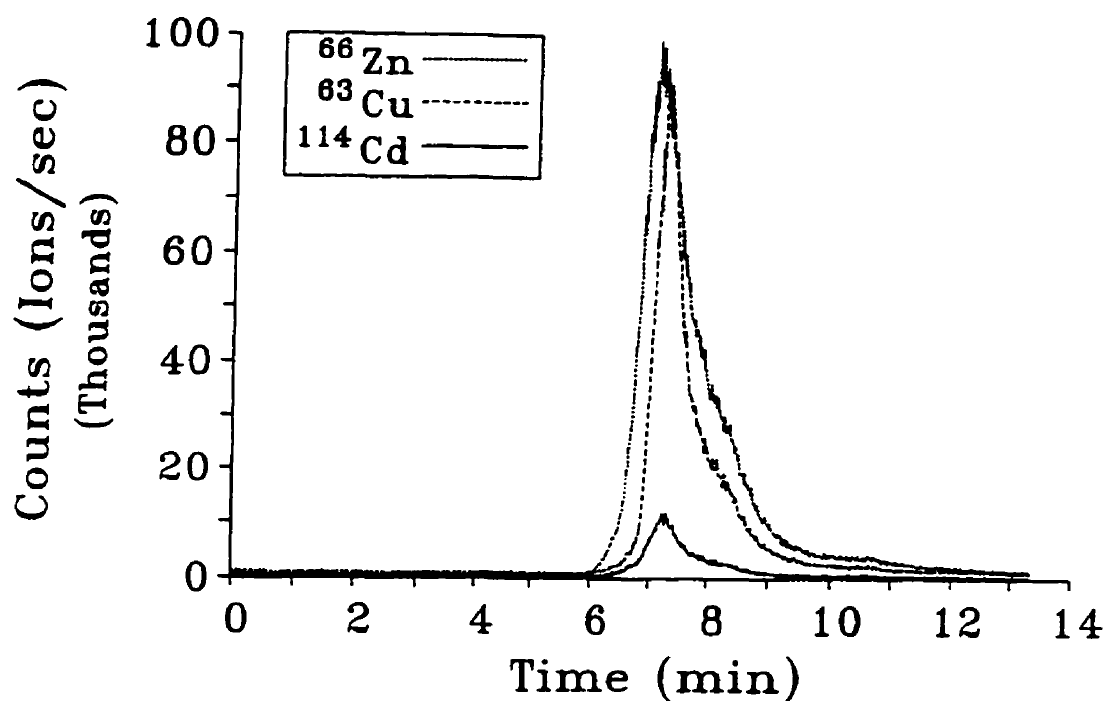


Figure 6.5. HPLC-ICP-MS chromatogram of a crude extract from a control *Dreissena* specimen showing the typical metal content of invertebrate metallothioneins; Cu, Zn, and Cd. The dimeric and monomeric forms (fig. 6.3) were not resolved in this instrumental configuration.

(Stillman *et al.*, 1987). Cuprous ions are also bound tightly by MT with a trigonal coordination geometry and a stoichiometry of 12:1 (Nielson *et al.*, 1985). The assumption of a fully reduced MT pool binding metals in a definite stoichiometry has been the basis of several quantitative metal binding assays that are based on the saturation of the thiolic complexing sites with a metallic marker [Ag^+ (Scheuhammer and Cherian, 1991); Cd^{2+} (Eaton and Cherian, 1991); and Hg^{2+} (Couillard *et al.*, 1993; Dutton *et al.*, 1993; Malley *et al.*, 1993)]. On the other hand, the cysteine residues are quite prone to oxidation by oxygen, trace metals (especially Cu(I)), heme compounds, etc. (Minkel *et al.*, 1980, Meister and Anderson, 1983). The result of this oxidation is the formation of intra- or intermolecular disulfide bridges (Suzuki and Yamamura, 1980) which cannot complex metals efficiently. Generally, metal saturation protocols do not include a reducing step to ensure that all cysteines in a MT extract are in the proper redox state for co-ordination. The authors recently found that a major proportion of the cysteine residues present in MT extracted in cold/anoxic conditions from *A. grandis grandis* were in the oxidized state (High *et al.*, 1995). Incubating *Anodonta* MT extracts with a reducing agent (mercaptoethanol) was an essential step to obtain a maximum uptake of Cd^{2+} ions by the polypeptide. The MT fraction extracted from zebra mussels responded to this reduction treatment as well (Figures 6.3 and 6.4).

With virtually no precedent on the *in vitro* saturation of invertebrate MT with vast excesses of Cd and a thiolic species, the exact stoichiometry of the Cd-MT complex formed under these conditions remains to be characterized. One

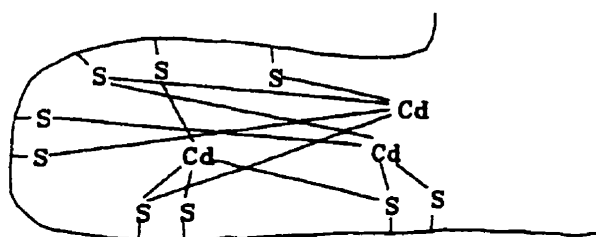
possible stoichiometry is the widely accepted cysteine-to-Cd ratio of 3:1 which is usually obtained by titration of apo-MT with Cd^{2+} (Figure 6.6a). However, this stoichiometry may not account for the relatively large amounts of Cd adsorbed by the MT in the presence of excess mercaptoethanol and for the additional uptake of free Cd^{2+} upon heating the treated samples for some time (Figure 6.4). Abnormal metal-MT stoichiometries of unknown biological significance have been observed via *in vitro* saturation of apo-MT, both divalent (Hg^{2+} , Lu *et al.*, 1993) and monovalent metals (Cu^+ , Ag^+ , Nielson *et al.*, 1985, Scheuhammer and Cherian, 1991). Thus, one likely Cd saturation mechanism would include the thiolic moieties of mercaptoethanol in the coordination structure (Figure 6.6b). The net results would be a reduction of the cysteine-to- Cd^{2+} ratio from 3:1 (Otvos and Armitage, 1980) in the Cd_7 -MT to 1:1, thus yielding a higher stoichiometric ratio for the Cd-MT complex. Although this structure would be less stable than the tetrahedral structure of the Cd_7 -MT complex, the possibility that a large excess of mercaptoethanol may effectively compete with MT cysteine residues for coordination of Cd^{2+} cannot be excluded and remains to be investigated.

6.6 Conclusions

The uncertain stoichiometry of the Cd-MT complex formed *in vitro* does not allow for an accurate determination of the absolute concentrations of MT in

the *Dreissena polymorpha* specimens. However, the fact that MT from all extracts adsorbed similar quantities of Cd^{2+} *in vitro* indicated that the physiological concentration of MT of all exposed specimens remained similar to the basal concentration in the control specimens. Thus, short-term exposure to environmentally relevant concentrations of Cd^{2+} did not produce a genetic induction of MT biosynthesis as generally observed in specimens exposed to higher concentrations of d^{10} metals. Under these experimental conditions, the physiological MT pool of *Dreissena* efficiently sequestered a large proportion of absorbed Cd^{2+} which could otherwise cause cellular damage. Additional studies are required to determine the Cd-MT stoichiometry and the minimum water Cd concentration and exposure time required to elevate the basal MT biosynthesis rate in *Dreissena polymorpha*.

A



B

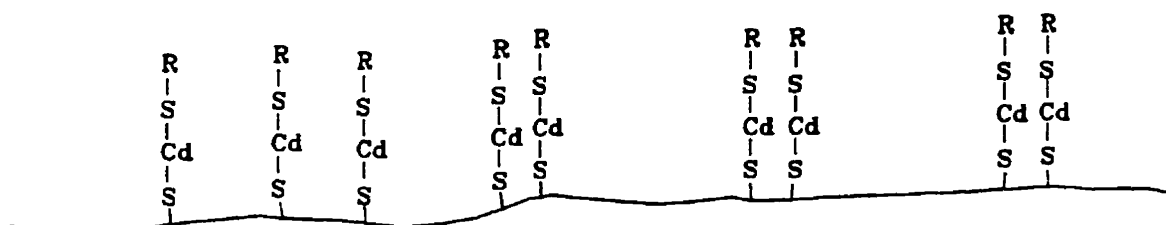


Figure 6.6. Possible coordination structure of the cadmium-saturated zebra mussel metallothionein: A) standard model, B) postulated model including mercaptoethanol in the structure.

6.7 References

- Bias, R., and L. Karbe. 1985. Bioaccumulation and partitioning of cadmium within the freshwater mussel *Dreissena polymorpha* Pallas. *Int. Rev. Gesamten Hydrobiol.* 70:113-125.
- Couillard, Y., P.G.C. Campbell, and A. Tessier. 1993. Response of metallothioneins in a freshwater bivalve (*Anodonta grandis grandis*) along an environmental cadmium gradient. *Limnol. Oceanogr.* 38:299-313.
- Dameron, C.T., G.N. George, P. Arnold, V. Santhanagopalan, and D.R. Winge. 1993. Distinct metal binding configurations in ACE1. *Biochemistry* 32:7294-7301.
- de Kock, W. Chr., and C.T. Bowmer. 1993. Bioaccumulation, biological effects, and food chain transfer of contaminants in the zebra mussel (*Dreissena polymorpha*). In: T. F. Nalepa, and D.W. Schloesser (Eds). Zebra mussels: Biology, impacts, control. Lewis Publ. NY. Pp. 491-502.
- Dion, B., M. Ruzbie, F.R. van de Voort, A.A. Ismail, and J.S. Blais. 1994. Determination of protein and fat in meat by transmission Fourier transform infrared spectrometry. *Analyst* 119:1765-1771.
- Dutton, M.D., M. Stephenson, and J.F. Klaverkamp. 1993. A mercury saturation assay for measuring metallothionein in fish. *Environ. Toxicol. Chem.* 12:1193-1202.

- Eaton, D.L. and M.G. Cherian,. 1991. Determination of metallothioneins in tissues by cadmium-hemoglobin affinity assay. *Methods Enzymol.* 205:83-87.
- Engel, D.W. and M. Brouwer. 1989. Metallothionein and metallothionein-like proteins: physiological importance. *Adv. Comp. Environ. Physiol.* 5:53-75.
- Hamburger K., P.C. Dall, and P.M. Jonasson. 1990. The role of *Dreissena polymorpha* Pallas (mollusca) in the energy budget of Lake Esrom, Denmark. *Verh. Internat. Verein. Limnol.* 24:621-625.
- Hebert, P.D.M., B.W. Muncaster, and G.L. Mackie. 1989. Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusk in the Great Lakes. *Can. J. Aquat. Fish Sci.* 46:1587-1591.
- Hemelraad, J., D.A. Holwerda, K.J. Teerds, H.J. Herwig, and D.I. Zandee. 1986. Cadmium kinetics in freshwater clams. II. A comparative study of cadmium uptake and cellular distribution in the Unionidae *Anodonta cygnea*, *Anodonta anatina*, and *Unio pictorum*. *Arch. Environ. Contam. Toxicol.* 15:9-21.
- Herwig, H.J., Brands, F., Kruitwagen, E., Zandee, D.I., (1989). Bioaccumulation and histochemical localization of cadmium in *Dreissena polymorpha* exposed to cadmium chloride. *Aquatic Toxicol.* 15, 269-286.
- High, K.,R. Azani., A.F. Fazekas, Z. Chee,. and J.S. Blais,. 1992. Determination of trace cadmium and cadmium-metallothioneins in biological samples with flow injection- and HPLC-atomic absorption spectrometry. *Anal. Chem.*, 64:3187-3201.

- High, K.A., B.A.Methven, J.W. McLaren, K.W.M. Siu, J. Wang, J.F. Klaverkamp, and J.S. Blais. 1995. Physico-chemical characterization of metal binding proteins using HPLC-ICP-MS, HPLC-MA-AAS and electrospray-MS. *Fresenius J. Anal. Chem.* 351:393-402.
- Griffiths, R.W., D.W. Schloesser, J.H. Leach, and W.P. Kovalak, P. 1991. Distribution and dispersal of the zebra mussel (*Dreissena polymorpha*) in the Great Lakes Region. *Can. J. Aquat. Fish Sci.* 48:1381-1388.
- Kagi, J.H.R. and A. Schaffer. 1988. Biochemistry of Metallothioneins. *Biochemistry* 27:8509-8515.
- Kraak, M.H.S., D. Lavy, W.H.M. Peeters, and C. Davids. 1992. Chronic ecotoxicity of copper and cadmium to the zebra mussel *Dreissena polymorpha*. *Arch. Environ. Contam. Toxicol.* 23:363-369.
- Kraak, M.H.S., M.C. Scholten, W.H.M. Peeters, and W. de Kock. 1991. Biomonitoring of heavy metals in the Western European rivers and Meuse using the freshwater mussel *Dreissena polymorpha*. *Environ. Poll.* 74:101-114.
- Kraak, H.S., D.L. Kraak, M. Toussaint, H. Schoon, W.H.M. Peeters, and C. Davids. 1993. Toxicity of heavy metals to the zebra mussel (*Dreissena polymorpha*). In: T.F. Nalepa and D.W.Schloesser (Eds). Zebra mussels: Biology, impacts, control. Lewis Publ., NY. Pp. 491-502.
- Labbé, S., L. Larouche, D. Mailhot, and C. Séguin. 1993. Purification of mouse MEP-1, a nuclear protein which binds to the metal regulatory elements of genes encoding metallothionein. *Nucleic Acids Research.* 21:1549-1554.

- Léglise, L. and C. Crochard. 1987. Vérification expérimentale du choix de *Dreissena polymorpha* Pallas (Lamellibranche) comme bioindicateur de contamination métallique. Nat. Can. (Rev. Ecol. Sys.) 114:315-323.
- Lu, W., A.J. Zelazowski, and M.J. Stillman. 1993. Mercury binding to metallothioneins: formation of the Hg₁₈-MT species. Inorg. Chem. 32:919-926.
- Mackay, E.A., J. Overnell, B. Dunbar, I. Davidson, P.E. Hunziker, H.R. Kagi, and J.E. Fothergill. 1993. Complete amino acid sequence of five dimeric and four monomeric forms of metallothioneins from the edible mussel *Mytilus edulis*. Eur. J. Biochem. 218:183-194.
- Malley, D.F., J.F. Klaverkamp, S.B. Brown, and P.S.S. Chang. 1993. Increase in metallothionein in freshwater mussels *Anodonta grandis grandis* exposed to cadmium in the laboratory and the field. Water Poll. Res. J. Canada:28, 253-273.
- Meister, A., and M E. Anderson. 1983. Glutathione. Ann. Rev. Biochem. 52:711-724.
- Mersch, J., E. Morhain, and C. Mouvet. 1993. Laboratory accumulation and depuration of copper and cadmium in the freshwater mussel *Dreissena polymorpha* and the aquatic moss *Rhynchostegium riparioides*. Chemosphere 27:1475-1485.
- Mills, E.L., E.F. Roseman, M. Rutzke, W.H. Gutenmann, and D.J. Lisk. 1993. Contaminants and nutrient element levels in soft tissues of zebra and

- quagga mussels from waters of Southern lake Ontario. *Chemosphere* 27:1465-1473.
- Minkel, D.T., K. Poulsen, S. Wielgus, C.F. Shaw, and D.H. Petering. 1980. On the sensitivity of metallothioneins to oxidation during isolation. *Biochem. J.* 191:475-485.
- Nielson, K.B., C.L. Atkin, and D.R. Winge. 1985. Distinct metal-binding configurations in metallothionein. *J. Biol. Chem.* 260:5342-5350.
- Otvos, J.D. and I.M. Armitage. 1980. Structure of the metal clusters in rabbit liver metallothionein. *Proc. Natl. Acad. Sci. USA* 77:7094-7098.
- Reeders, H.H., A.B.D. Vaate, and F.J. Slim. 1989. The filtration rate of *Dreissena polymorpha* (Bivalvia) in three Dutch Lakes with reference to biological water quality management. *Freshwater Biol.* 22:133-141.
- Roseijadi, G., S. Kielland, and P. Klerk. 1989. Purification and properties of novel molluscan metallothioneins. *Arch. Biochem. Biophys.* 273:403-412.
- Scheuhammer, A.M. and M.G. Cherian. 1991. Quantification of metallothioneins by silver saturation. *Methods Enzymol.* 205:78-82.
- Secor, C.L., E.L. Mills, J. Harshbarger, H.T. Kuntz, W.H. Gutenmann, and D.J. Lisk,. 1993). Bioaccumulation of toxicants, elements and nutrient composition, and soft tissue histology of zebra mussels (*Dreissena polymorpha*) from New York state waters. *Chemosphere* 26:1559-1575.
- Stillman, M.J., W. Cai, and A.J. Zelazowski. 1987. Cadmium binding to metallothioneins. *J. Biol. Chem.* 262:4538-4548.

Stillman, M.J., C. F. Shaw, K.T. Suzuki, and T. Kazuo (ed.). 1992.

Metallothioneins. VCH New York. 569 pp.

Suzuki, K.T. and M. Yamamura. 1980. Isolation and characterisation of metallothionein dimers. *Biochem. Pharmacol.* 29:689-692.

Vallee, B.L. 1991. Introduction to metallothioneins. *Methods Enzymol.* 205:3-7.

Viarengo, A., M. Pertica, G. Mancinelli, G. Zanicchi, and M. Orunesu. 1980.

Rapid induction of copper-binding proteins in the gills of metal exposed mussels. *Comp. Biochem. Physiol.* 67:215-218.

CHAPTER 7

SUMMARY AND FINAL CONCLUSIONS

We demonstrated that lesser scaup can be used as a model species to study predator-prey interactions in aquatic system. Diving ducks have infrequently been used as sentinel species because of incubation and rearing difficulties. We proposed techniques to enhance hatching success and improve survival of the ducklings. Methods were also described to keep the adults in outdoor pens. This work was essential to optimize the living conditions of the species and to establish population of lesser scaup in semi-natural conditions to enable the undertaking of the rest of the project.

We also developed or optimized methodologies to measure different biomarker responses. With the help of flow cytometry, the phagocytic activity and peroxidase production of heterophils were assessed for the first time. It was demonstrated that there was a decrease of the nonspecific immunity of lesser scaup feeding on zebra mussel which was positively correlated with the incidence of pododermatitis. Decreases in hepatic vitamin A and histopathological alterations were observed in the ducks fed zebra mussel from two different localities. These results suggested that zebra mussels may have deleterious impacts on the general health of lesser scaup, even when they are consumed only for a relatively short period of time.

In the last section of this thesis, the zebra mussel was assessed for its capacity to sequester cadmium. It was demonstrated that zebra mussel cannot regulate Cd at trace exposure concentration. Metallothionein biosynthesis was not induced at environmentally relevant concentration of Cd ²⁺ as is generally observed when animals are exposed to higher concentrations of heavy metals.

This study has contributed to our understanding of the immunological and biochemical responses of an organism confronted with a new, contaminated, food source. The use of a suite of biomarkers can: 1) enhance our understanding of the interactions and mechanisms of actions existing between the different biomarkers at the individual level, 2) verify and validate which biomarkers are more sensitive and applicable in field conditions at environmentally relevant exposure levels, 3) give valuable information on bioaccumulation and biomagnification impacts between predators and contaminated prey, 4) in the context of bioeffects monitoring, link biomarker responses to higher organizational level responses such as reproductive failure and epidemic outbreaks, and finally 5) be used as management tools as a predictor of population fluctuations in bird species.

Further work is necessary to link the results obtained in this project to higher levels of organization. Thus, research should be undertaken both in the wild and under semi-natural conditions. Bird surveys have revealed a diminution in the reproductive success of lesser scaup in the wild for the past three seasons (J. Barclay, pers. comm.). It would be interesting to test if this reduction could be correlated with the biomarkers studied in the present project. Furthermore,

similar experiments with lesser scaup held in semi-natural conditions could incorporate a behavioral or reproduction component to assess the possible interactions between “early” biochemical, immunological and histopathological biomarkers and population or community biomarkers.

REFERENCES

- Abbas, A.K., A.H. Lichtman, and J.S. Pober. 1994. Cellular and molecular immunology. W.B. Saunders Co., Philadelphia, PA. 457 pp.
- Akins, J.M., M.J. Hooper, H. Miller, and J.S. Woods. 1993. Porphyrin profiles in the nestling European starling (*Sturnus vulgaris*): a potential biomarker of field contaminant exposure. J. Toxicol. Environ. Health 40:47-59.
- Bernard, R.F. 1963. Studies of the effects of DDT on birds. Michigan State University Mus. Pub. Biol. Ser. 2(3):155-192.
- Bitman, J., H.C. Cecil and S.J. Harris. 1969. DDT induces a decrease in eggshell calcium. Nature 224:44-46.
- Boersma, D.C., J.A. Ellenton and A. Yagminas. 1986. Investigation of the hepatic mixed-function oxidase system in herring gull embryos in relation to environmental contaminants. Environ. Toxicol. Chem. 5:309-318.
- Braunbeck, T.A. S.J. Teh, S.M. Lester, D.E. Hinton. 1992. Ultrastructural alterations in liver of medaka (*Oryzias latipes*) exposed to diethylnitrosamine. Toxicol. Pathol. 20:179-196.
- Brieger, G. and R.D. Hunter. 1993. Uptake and depuration of PCB 77, PCB 169, and hexachlorobenzene by zebra mussels (*Dreissena polymorpha*). Ecotoxicol. Environ. Safety 26:153-165.
- Brouwer, M., D.R. Winge, and W.R. Gray. 1989. Structural and functional diversity of copper-metallothionein from the American Lobster, *Homarus americanus*. J. Inor. Biochem. 35:289-303.

- Brunet, S., F. Guertin, D. Flipo, M. Fournier, and K. Krzystyniak. 1993. Cytometric profiles of bone marrow and spleen lymphoid cells after mercury exposure in mice. *Int. J. Immunopharmacol.* 15:811-819.
- Cade, T.J., J.H. Enderson, C.G. Thelander, and C.M. White (Eds). 1988. Peregrine falcon populations: their management and recovery. The Peregrine Fund, Inc. Boise, ID. 949 pp.
- Carpenter, H.M., D.E. Williams, M.C. Henderson, R.D. Bender, and D.R. Buhler. 1984. Hexachlorobenzene-induced porphyria in Japanese quail. *Biochem. Pharmacol.* 33:3875-3881.
- Chen, S.W., P.J. Dziuk, and B. M. Francis. 1994. Effect of four environmental toxicants on plasma Ca and estradiol 17 and hepatic P450 in laying hens. *Environ. Toxicol. Chem.* 13:78-796.
- Contrino, J., L.L. Kosuda, P. Marucha, D.L. Kreutzer, and P.E. Bigazzi. 1992. The *in vitro* effects of mercury on peritoneal leucocytes (PMN and macrophages) from inbred Brown Norway and Lewis rats. *Int. J. Immunopharmacol.* 14:1051-1059.
- Coombs, T.L., 1979. Cadmium in aquatic organisms. In: M. Webb (Ed.). The chemistry, biochemistry and biology of cadmium. Elsevier, Amsterdam. Pp. 92-206.
- Couillard, Y., P.G.C. Campbell, and A. Tessier. 1993. Response of metallothionein concentrations in a freshwater bivalve (*Anodonta grandis*) along an environmental cadmium gradient. *Limnol. Oceanogr.* 38:299-313.

- Cronan, J.M. Jr. 1957. Food and feeding habits of the scaups in Connecticut waters. *Auk* 74:459-468.
- Custer, C.M., 1993. Third International Zebra Mussel Conference, Feb. 23-26 1993, Toronto, Canada.
- Day, R.S., L. Eales, and P.B. Disler. 1981. Porphyrias and the kidney. *Nephron* 28:261-267.
- de Koch, W.Ch, and C.T. Bowmer. 1993. Bioaccumulation, biological effects, and food chain transfer of contaminants in the zebra mussel (*Dreissena polymorpha*). In: Nalepa, T.F. and D.W. Schloesser (Eds), Zebra mussels: Biology, Impacts, and control. Lewis publishers, Florida, USA. p. 503-533.
- Depledge, M. H., and S.P. Hopkin. 1995. Methods to assess effects on brakish, estuarine, and near-coastal water organisms. In: Linthurst, R.A., P. Boudreau and R.G. Tardiff (Eds), Methods to assess the effects of chemicals on ecosystems. John Wiley & Sons Ltd, pp.125-149.
- Depledge, M.H., J.J. Amaral-Mendes, B. Daniel, R.S. Halbrook, P. Kloepper-Sams, M.N. Moore, and D.B. Peakeall. 1993. The conceptual basis of the biomarkers approach. In: D.B. Peakall, and L.R. Shugart (eds). Biomarker. NATO ASI series, vol H 68, Springer-Verlag, Berlin, pp. 15-29.
- Dermott, R., J. Leslie, J. Fitzsimmons and V. Cairns, 1990. Proceeding of the International Zebra Mussel research conference, Dec. 5-7 1990, Ohio Sea Grant College Program- The Ohio State University, 29 p.

- Dieter, M.P. 1974. Plasma enzyme activities in coturnix quail fed graded doses of DDE, polychlorinated biphenyl, malathion, and mercuric chloride. *Toxicol. Appl. Pharmacol.* 27:86-98.
- Dion, J., and Y. Richer, 1994. Dynamique des populations de Dreissenids dans le St-Laurent: perspectives pour orienter les objectifs de contrôle en industries. Troisième conférence provinciale sur la moule zébrée, 16 mars 1994, Québec.
- Dixon, K.C. 1982. Cellular defects in disease. Blackwell Scientific Publications, Oxford, England. 476 pp.
- Doherty, F.G., D.W. Evans and E.F. Neuhauser. 1993. An assessment of total and leachable contaminants in zebra mussels (*Dreissena polymorpha*) from Lake Erie. *Ecotoxicol. Environ. Safety* 25:328-340.
- Eisler, R, and J. Jacknow. 1985. Toxaphene hazards to fish, wildlife and invertebrates: a synoptic review. U.S. Fish and Wildlife Service Contam. Hazard Rev. 4:5-11.
- Engel, D.W., and M. Bouwer. 1989. Metallothionein and metallothionein-like proteins: physiological importance. *Adv. Comp. Environ. Physiol.* 4:53-75.
- Faith, R.E. , M.I. Luster, and J.G. Vos. 1980. Effects of immunocompetence by chemicals of environmental concern. *Rev. Biochem. Toxicol.* 2:173-211.
- Fox, G.A., A.P. Gilman, D.P. Peakall, and F.W. Anderka. 1978. Behavioral abnormalities of nesting Lake Ontario herring gulls. *J. Wildl. Manage.* 42:477-483.

- Fox, G.A. 1993. What have biomarkers told us about the effects of contaminants on the health of fish-eating birds in the Great Lakes? The theory and a literature review. *J. Great Lakes Res* 19:722-736.
- Fox, G.A., P. Mineau, B. Collins, and P.C. James. 1989. The impact of the insecticide carbofuran (Furadan 480F) on the Burrowing Owl in Canada. Tech. Rep. Ser. No. 72., Canadian Wildlife Service, Ottawa. 25 pp.
- Fox, G.A., S.W.Kennedy, R.J. Nostrom and D.C. Wigfield. 1988. Porphyria in herring gulls: a biochemical response to chemical contamination of the Great Lakes food chain. *Environ. Toxicol. Chem.* 7:831-839.
- French III, J.P.R. and M.T. Bur. 1993. Predation of the zebra mussel (*Dreissena polymorpha*) by freshwater drum in Western Lake Erie. In: Nalepa, T.F. and D.W. Schloesser (Eds), Zebra mussels: Biology, Impacts, and Control. Lewis Publishers, Florida, USA. p. 453-464.
- Friend, M., and D.D. Trainer. 1974. Experimental dieldrin-duck hepatitis virus interaction studies. *J. Wildl. Manag.* 38:896-902.
- Gardner, G.R., R.J. Pruell, and L.C. Folmar. 1989. A comparison of both neoplastic and non-neoplastic disorders in winter flounder (*Pseudopleuronectes americanus*) from eight areas in New England. *Mar. Environ. Res.* 28:393-397.
- Gauthier, J. and Y. Aubry. 1995. Les oiseaux nicheurs du Québec. Montreal. Pp. 312-315.

- Géroutet, P. 1966. Premières conséquences ornithologiques de l'introduction de la moule zébrée *Dreissena polymorpha* dans le lac Léman. Nos Oiseaux 28:301-307.
- Gilbertson, M. 1975. A Great Lakes tragedy. Nat. Can. 4:22-25.
- Gilbertson, M. and G.A. Fox. 1977. Pollutant-associated embryonic mortality of Great Lakes Herring gulls. Environ. Pollut. 12:211-216.
- Goven, A.J., B.J. Venables, L.C. Fitzpatrick, and E.L. Cooper. 1988. An invertebrate model for analyzing effects of environmental xenobiotics on immunity. Clin. Ecol. 4:150-154.
- Government of Canada. 1991. Toxic chemicals in the Great Lakes and associated effects, vol. II-Effects. Supply and Services Canada.
- Hamburger., K., P.C. Dall, and P.M. Jonasson. 1990. The role of *Dreissena polymorpha* Pallas (Mollusca) in the energy budget of Lake Esrom, Denmark. Verh. Int. Verein. Theor. Angew. Limnol. 24:621-625.
- Hamilton, D.J. 1992. A method for reconstruction of zebra mussel (*Dreissena polymorpha*) length from shell fragments. Can. J. Zool. 70:2486-2490.
- Hamilton, D.J., C.D. Ankney, and R.C. Bailey. 1994. Predation of zebra mussels by diving ducks: an exclosure study. Ecology 75:521-531.
- Hayes, M.A., I.R. Smith, T.H. Rushmore, T.L. Crane, C. Thorn, T.E. Kocal, and H.W. Ferguson. 1990. Pathogenesis of skin and liver neoplasms in white suckers from industrially polluted areas in Lake Ontario. Sci. Total Environ. 94:105-123.

- Hebert, P.D.N., B.W. Muncaster, and G.L. Mackie, 1989. Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusc in the Great Lakes. Can. J. Fish. Aquat. Sci. 46:1587-1591.
- Hickey, J.J. 1969. The peregrine falcon populations: the biology and decline. University of Wisconsin Press, Madison, WI. 596 pp.
- Hinton, D.E., and D.J. Laurén. 1990. Liver structural alterations accompanying chronic toxicity in fishes: potential biomarkers of exposure. In: J. McCarthy, and L.R. Shugart (Eds.) Biological markers of environmental contamination. CRC Press, Boca Raton, FL.
- Hoffman, D.J., B.A. Rattner, C.M. Bunck, A. Krynisky, H.M. Ohlendorf, and R.W. Lowe. 1986. Association between PCBs and lower embryonic weight in black-crowned night herons in San Francisco Bay. J. Toxicol. Environ. Health 19:393-391.
- Hoffman, D.J., B.A. Rattner, L. Sileo, D. Docherty and T.J. Kubiak. 1987. Embryotoxicity, teratogenicity, and aryl hydrocarbon hydroxylase activity in Foster's terns on Green Bay, Lake Michigan. Environ. Res. 42:176-184.
- Hoffman, D.J., B.A. Rattner, L. Sileo, D. Docherty, and T.J. Kubiak. 1987. Embryotoxicity, teratogenicity and aryl hydrocarbon hydroxylase activity in Forster's terns on Green Bay, Lake Michigan. Environ. Res. 42:176-184.
- Huggett, R.J., R.A. Kimerle, P.M. Mehrle, and H.L. Berman. 1992. Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. Lewis Publishers, Florida, USA. 347p.

- Kagi, J.H.R., and A. Schaffer. 1988. Biochemistry of metallothioneins. *Biochemistry* 27:8509-8515.
- Kennedy, S.W., and C.A. James. 1993. Improved method to extract and concentrate porphyrins from liver tissue for analysis by high-performance liquid chromatography. *J. Chromatogr.* 619:127-132.
- Kennedy, S.W., and S.P. Jones. 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem* 222:217-223.
- Klaunig, J.E., M.M. Lipsky, B.F. Trump, and D.E. Hinton. 1979. Biochemical and ultrastructural changes in teleost liver following subacute exposure to PCB. *J. Environ. Pathol. Toxicol.* 2:953-963.
- Knight, G.C., and C.H. Walter. 1982. A study of the hepatic microsomal monooxygenase of sea birds and its relationship to organochlorine pollutants. *Comp. Biochem. Physiol.* 73C:211-221.
- Koller, L.D. 1977. Enhanced polychlorinated biphenyl lesions in Moloney leukaemia virus-infected mice. *Clin. Toxicol.* 11:107-116.
- Koller, L.D. 1979. Effects of environmental contaminants on the immune system. *Adv. Vet. Sci. Comp. Med.* 23:267-295.
- Koller, L.D. 1979. Effects of environmental contaminants on the immune system. *Adv. Vet. Sci. Comp. Med.* 23:267-295.
- Koller, L.D., J.G. Roan, and N.I. Kerkvliet. 1979. Mitogen stimulation of lymphocytes in CBA mice exposed to lead and cadmium. *Environ. Res.* 19:177-188.

- Koss, G, E.S. Schuler, B. Arndt, J. Seidel, S. Seubert, and A. Seubert. 1986. A comparative toxicological study of pike (*Esox lucius* L.) from the River Rhine and River Lahn. *Aquat Toxicol.* 8:1-9.
- Kraak, M.H.S., M.C.T. Scholten, W.H.M. Peeters and W.C. de Kock. 1991. Biomonitoring of heavy metals in the western European rivers Rhine and Meuse using the freshwater mussel *Dreissena polymorpha*. *Environ. Pollut.* 74:101-114.
- Kreis, R.G., Jr., M.D. Mullin, R. Rossman and L.L. Wallace. 1991. Organic contaminant and heavy metal concentrations in zebra mussel tissue from western Lake Erie. 2nd International Zebra Mussel Research Conference, November 19-22, Rochester, N.Y.
- Krzystyniak, K., P. Hugo, D. Flipo, M. Fournier. 1985. Increased susceptibility to mouse hepatitis virus 3 of peritoneal macrophages exposed to dieldrin. *Toxicol. Appl. Pharmacol* 80:397-408.
- Krzystyniak, K., B. Trottier, P. Jolicoeur, and M. Fournier. 1987. Macrophage functional activities versus cellular parameters upon sublethal exposure in mice. *Mol. Toxicol.* 1:247-259.
- Labbé, S. L. Larouche, D. Mailhot, and C. Séguin. 1993. Purification of mouse MEP-1, a nuclear protein which binds to the metal regulatory elements of genes encoding metallothionein. *Nucleic Acids Res.* 21:1549-1554.
- Landrum, P.F., D.C. Gossiaux, S.W. Fisher, and K.A. Bruner. 1990. The role of zebra mussels in contaminant cycling in the Great Lakes. 1st International

Zebra mussel Research Conference, December 5-7, Ohio State University, Ohio.

- Léglise, L. et C. Crochard. 1987. Vérification expérimentale du choix de *Dreissena polymorpha pallas* (Lamellibranche) comme bioindicateur de contamination métallique. *Naturalliste Can. (Rev. Écol. Syst.)* 114:315-323.
- Lewis, L.D., M.L. Morris and M.S. Hand. 1987. Alimentation clinique des petits animaux III. Mark Morris Associates (Eds), Topeka, Texas.
- Longcore, J.R., and G.W. Cornwell. 1964. The consumption of natural foods by captive canvasbacks and lesser scaups. *J. Wildl. Manage.* 28:527-531.
- Loose, L.D., J.B. Silkworth, and D.W. Simpson. 1978. Influence of cadmium on phagocytic and microbicidal activity of murine peritoneal macrophages, pulmonary alveolar macrophages, and polymorphonuclear neutrophils. *Infect. Immun.* 22:378-381.
- Lu, F.C. 1991. Basic Toxicology. Fundamentals, target organs, and risk assessment. Hemisphere Publ. Co. U.S 361p.
- MacIsaac, H.J., W.G. Sprules, O.E. Johannsson and J.H. Leach. 1992. Filtering impacts of larval and sessile zebra mussels (*Dreissena polymorpha*) in western Lake Erie. *Oecologia* 92:30-39.
- Maines, M.D. 1984. New developments in the regulation of heme metabolism and their implication. *CRC Crit. Rev. Toxicol.* 12:241:311.

- Marks, G.S., D.T. Zelt, and S.P.C. Cole. 1982. Alterations in the heme biosynthesis pathway as an index of exposure to toxins. *Can. J. Physiol. Pharmacol.* 60:1017-1026.
- Mayer, F.L., D.J. Versteef, M.J. McKee, L.C. Folmar, R.L. Graney, D.C. McCume, and B.A. Rattner. 1992. Physiological and nonspecific biomarkers. In: Huggett, R.J., R.A. Kimerle, P.M. Mehrle, and H.L. Berman (Eds). Biomarkers:biochemical, physiological, and histological markers of anthropogenic stress. Lewis Publishers, Florida, USA. Pp. 5-85.
- McArthur, M.L.B., G.A. Fox, D.B. Peakall, and B.J.R. Philogène. 1983. Ecological significance of behavioral and hormonal abnormalities in breeding ring doves fed an organochlorine chemical mixture. *Arch. Environm. Contam. Toxicol.* 12:343-353.
- McCarthy, J.F., and L.R. Shugart. 1990. Biological markers of environmental contamination In: J.F. McCarthy and L.R. Shugart (Eds). Biomarkers of environmental contamination. Lewis Publishers, Boca Raaton, FL. Pp.3-14.
- Mellina, E and J.B. Rasmussen. 1994. Patterns in the distribution and abundance of zebra mussel (*Dreissena polymorpha*) in rivers and lakes in relation to substrate and other physicochemical factors. *Can. J. Fish. Aquat. Sci.* 51:1024-1036.
- Meyers, T.R., and J.D. Hendricks. 1985. Histopathology. In: G.M.. Rand, and S.R. Petrocelli (Eds.). Fundamentals of aquatic toxicology. Hemisphere Publishing Corp., Washington, D.C. pp. 283-331.

- Mineau, P. 1991. The regulatory assessment of cholinesterase-inhibiting insecticides. In Mineau, P. (Ed.), Cholinesterase-inhibiting insecticides-their impact on wildlife and the environment. Elsevier, Amsterdam. pp. 277-300.
- Miranda, C.L., M.C. Henderson, J.-L. Wang, H.S. Nakae, and D.R. Buhler. 1987. Effects of polychlorinated biphenyls on porphyrin synthesis and cytochrome P-450-dependent monooxygenases in small intestine and liver of Japanese quail. *J. Toxicol. Environ. Health* 20:27-35.
- Miranda, C.L., M.C. Henderson, J.-L. Wang, H.S. Nakae, and D.R. Buhler. 1992. Comparative effects of the polychlorinated biphenyl mixture, Aroclor 1242, on porphyrin and xenobiotic metabolism in kidney of Japanese quail and rat. *Comp. Biochem. Physiol.* 103C:149-152.
- Mitchell, C.A. and J. Carlson. 1993. Lesser scaup forage on zebra mussels at Cook nuclear plant, Michigan. *J. Field Ornithol.* 64:219-222.
- Myers, M.S., J.T. Landahl, M.M. Krahnn, L.L. Johnson, and B.B. McCain. 1990. Overview of studies on liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology: pathology and epizootiology. *Sci. Total Environ.* 94:33-50.
- National Research Council (NRC). 1987. Committee on biological markers. *Environ. Health Perspect.* 74:3-9.
- National Research Council of Canada (NRCC). 1981. Polychlorinated dibenzo-p-dioxins. Criteria for their effects on man and his environment. Ottawa.

- New York Sea Grant, 1994. North American range of the zebra mussel. In O'Neil, C. (ed.). Dreissenal 5:6-7. Published by the Zebra Mussel Information Clearinghouse.
- New York Sea Grant. 1996. Distribution map of zebra mussels in North America.
- Noël-Lambot, F, J.M. Bouquegneau, F. Frankenne, and A. Disteche. 1980. Cadmium, zinc and copper accumulation in limpets (*Patella vulgata*) from the Bristol Channel with special reference to metallothioneins. Mar. Ecol. Prog. Ser. 2:81-89.
- O'Brien, R.D. 1967. Insecticides: action and metabolism. Academic Press. New York. 126 pp.
- Oh, S.H., J.T. Deagen, P.D. Whanger, and P.H. Weswig. 1978. Biological function of metallothionein. V. Its induction in rats by various stresses. Am. J. Physiol. 3:282-285.
- Olson, J.A. 1981. Formation and function of vitamin A. In: J.W. Porter, and S.L. Spurgeon (Eds). Biosynthesis of isoprenoid compounds. John Wiley & Sons, New York. Pp. 371-412.
- Parkinson, A., S. Safe, L. Robertson, P.E. Thomas, D.E. Ryan, L.M. Reik, and E. Levin. 1983. Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated and polybrominated biphenyls: a study of structure activity relationships. J. Biol. Chem. 258:5967.
- Peakall, D.B. 1967. Pesticide-induced breakdown of steroids in birds. Nature 216:505-506.

- Peakall, D.B. 1970. P,p'DDT: effect on calcium metabolism and concentration of estradiol in the blood. *Science* 168:592-594.
- Peakall, D.B., R.J. Norstrom, A.D. Rahimtula, and R.D. Butler. 1986. Characterization of mixed-function oxidase systems of the nestling herring gull and its implications for bioeffects monitoring. *Environ. Toxicol. Chem.* 5:379-385.
- Pedroli, J.C. 1981. Les relations entre la moule zébrée *Dreissena polymorpha* (Pallas) et les oiseaux aquatiques. Dissertation. Université de Neuchâtel, Neuchâtel, Switzerland.
- Peterle, T.J. 1991. Wildlife Toxicology. Van Nostrand Reinhold, New York. 322 pp.
- Peters, G., T. Lang, M. Faisal, and I. Ahmed. 1990. The relationship of stress produced in culture to the natural defense mechanisms against disease. In: N. Fijan (Ed.). *Proceeding of the international symposium on ichthyopathology in aquaculture*. Yugoslavia Academy of Science.
- Pitot, H.C. 1988. Hepatic neoplasia: chemical induction. In: I.M. Arias, W.B. Jakoby, and H. Popper (Eds). The liver: biology and pathology. Raven Press, New York. Pp. 1125-1146.
- Prejs, A., K. Lewandowski, and A. Stanczykowska-Piotrowska, 1990. Size-selective predation by roach (*Rutilus rutilus*) on zebra mussel (*Dreissena polymorpha*): field studies. *Oecologia* 83:378-384.
- Rainbow, P.S., and R. Dalliger. 1993. Metal uptake, regulation, and excretion in freshwater invertebrates. In: Rainbow, P.S., and R. Dalliger (eds).

Ecotoxicology of metals in invertebrates. Lewis publishers, Boca Raton, FL. Pp119-131.

Ramshaw, J.A.M., B.J. Rigby, T.W. Mitchell and A. Nieass. 1986. Changes in the physical properties of skin collagen from broiler chickens exhibiting oily bird syndrome. Poultry. Sci. 65:43-50.

Rand, A.L. 1971. Birds of North America. Doubleday, New York, 255pp.

Rattner, B.A., D.J. Hoffman and C.M. Marn. 1989. Use of mixed-function oxygenases to monitor contaminant exposure in wildlife. Environ. Toxicol. Chem. 8:1093-1102.

Reynoldson, T.B., D.W. Schloesser, and B.A. Manny. 1989. Development of a benthic invertebrate objective for mesotrophic Great Lakes waters. J. Great Lakes Res. 15:669-696..

Ricciardi, A., F.G. Whoriskey, and J.B. Rasmussen. 1995. Predicting the intensity and impact of *Dreissena* infestation on native unionid bivalves from *Dreissena* field density. Can. J. Fish. Aquat. Sci. 52:1449-1461.

Richie, P.J., and T.J. Peterle. 1979. Effects of DDE on circulating luteinizing hormone levels in ring doves during courtship and nesting. Bull. Environ. Contam. Toxicol. 23:220-226.

Roberts, A.B., and M.B. Sporn. Cellular biology and biochemistry of the retinoids. In: M. B. Sporn, A.B. Roberts and D.S. Goodman (eds), The Retinoids, vol 2. Academic Press, Toronto, Canada, pp.210-287.

Rocke, T.E., T.M. Yuill, and R.D. Hinsdill. 1984. Oil and related toxicant effects on mallard immune defenses. Environ. Res. 33:343-352.

- Rodgers, J.P., and L.J. Korschgen. 1966. Foods of lesser scaups on breeding, migration and wintering areas. *J. Wildl. Manag.* 30:258-264.
- Roesijadi, G. 1981. The significance of low molecular weight, metallothionein-like proteins in marine invertebrates: current status. *Mar. Environ. Res.* 4:167-179.
- Roesijadi, G. 1982. Uptake and incorporation of mercury into mercury-binding proteins of gills of *Mytilus edulis* as a function of time. *Mar Biol.* 66:151-157.
- Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat. Toxicol.* 22:81-114.
- Rogers, J. P., and Korschgen, L.J. 1966. Food of lesser scaups on breeding, migration, and wintering areas. *J. Wildl. Manag.* 30:259-265.
- Safe, S. 1984. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, toxicology, and mechanism of action. *Crit. Rev. Toxicol.* 13:319-395.
- Sano, S. S. Kawanishi and Y. Seki. 1985. Toxicity of polychlorinated biphenyl with special reference to porphyrin metabolism. *Environ. Health Perspect.* 59:137-143.
- Schmidt, E. 1975. Die Ergebnisse der ersten November-Entenzahlung in der VR Ungarn. *Der Falke* 22:88-91.
- Scholten, M.C.T., E. Foekema, W.C. de Kock and J.M. Marquenie, 1989. Proc. 2nd European Symposium on Avian Medicine and Surgery, 8-11 March 1989, Utrecht, The Netherlands.

- Serrouya, R., A. Ricciardi, and F.G. Whoriskey. 1995. Predation on zebra mussels (*Dreissena polymorpha*) by captive-reared map turtles (*Graptemys geographica*). *Can. J. Zool.* 73:2238-2243.
- Snyder, F.L. 1990. Fact sheet O45, Ohio Sea Grant College Program, Columbus 4 p.
- Snyder, R.L. and J. Terry. 1986. Avian nutrition. In: Zoo and wild animal medicine. M.E. Fowler (eds), Toronto.
- Sommer, A. 1990. Vitamin A status, resistance to infection, and childhood mortality. *Ann. N.Y. Acad. Sci.* 587:17-23.
- Spear, P.A. D.H. Bourbonnais, D.B. Peakall and, T.W. Moon,. 1989. Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3',4,4'-tetrachlorobiphenyl. *Can. J. Zool.* 67:908-911.
- Spear, P.A. T.W. Moon, D.B. Peakall. 1986. Liver retinoid concentrations in natural populations of herring gull (*Larus argentatus*) contaminated by 2,3,7,8-tetrachlorodibenzo-p-dioxin and in ring doves (*Streptopelia risoria*) injected with a dioxin analogue. *Can. J. Zool.* 64:204-208.
- Spear, P.A., D.H. Bourbonnais, R.J. Norstrom, and T.W. Moon. 1990. Yolk retinoids (Vitamin A) in eggs of the herring gull and correlations with polychlorinated dibenzo-p-dioxins and dibenzofurans. *Environ. Toxicol. Chem.* 9:1053-1061.
- Spung, M. and U. Rose. 1988. Influence of food size quantity on the feeding of the mussel *Dreissena polymorpha*. *Oecologia* 77:526-532.

- Sregeman, J.J., R.L. Binder, and A. Orren. 1979. Hepatic and extrahepatic microsomal electron transport components and mixed-function oxygenases in the marine fish *Stenotomus versicolor*. *Biochem. Pharmacol.* 28:3431-3439.
- Stanczykowska, A. 1977. Ecology of *Dreissena polymorpha* (Pall.) (Bivalvia) in lakes. *Pol. Arch. Hydrobiol.* 24:461-530.
- Stanczykowska, A., P. Zyska, A. Dombrowska, H.Kot, and E. Zyska. 1990. The distribution of waterfowl in relation to mollusc populations in the man-made Lake Zegrzynskie. *Hydrobiologia* 191:233-240.
- Stegeman, J.J., M. Brouwer, R.T. Di Giulio, L. Forlin, B.A. Fowler, B.M. Sanders, and P.A. Van Veld. 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett, R.J., R.A. Kimerle, P.M. Mehrle, and H.L. Berman.. Biomarkers:biochemical, physiological, and histological markers of anthropogenic stress. Lewis Publishers, Florida, USA. Pp 235-335.
- Stehr, C. 1990. Ultrastructure of vacuolated cells in the liver of rock sole and winter flounder living in contaminated environment. *Proceedings of the 12th international congress for electron microscopy*. Pp. 522-523.
- Strayer, D.L., 1991. Projected distribution of zebra mussel, *Dreissena polymorpha* in North America. *Can. J. Fish. Aquat. Sci.* 48:1389-1395.

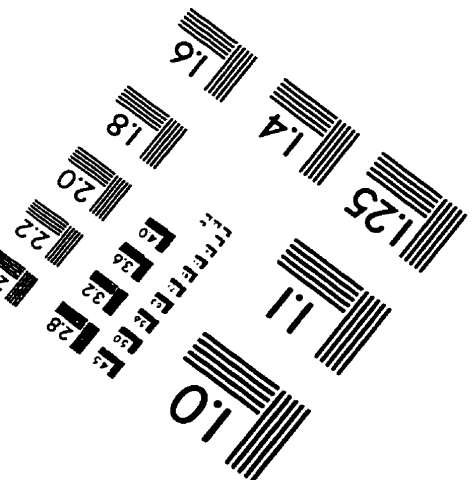
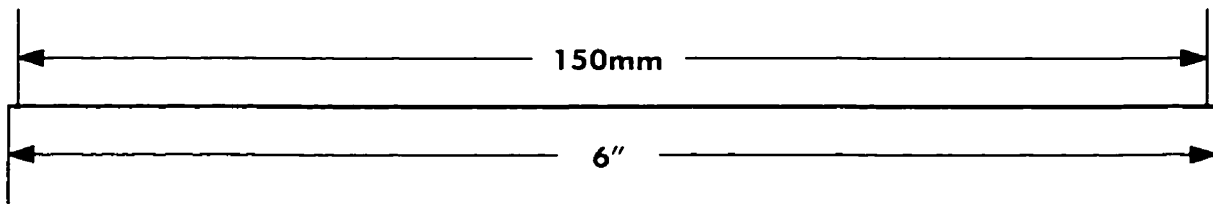
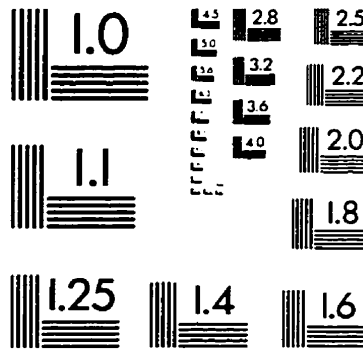
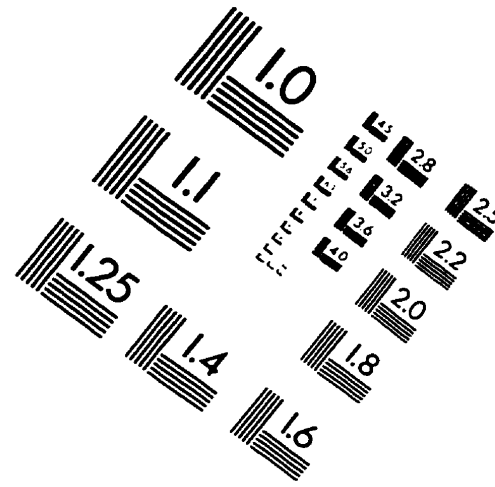
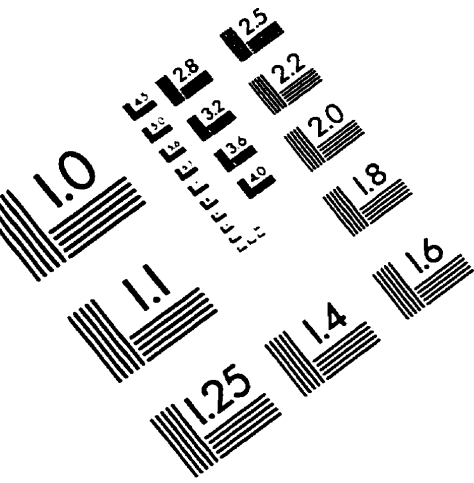
- Suter, W. 1982. Vergleichende Nahrungsökologie von überwinternden Tauchenten (*Bucephala*, *Aythya*) und Blabhn (*Fulica atra*) am Untersee-Ende/Hochrhein (Bodensee). Der Ornithologische Beobachter 79:225-24.
- Terres, J.K. 1987. Encyclopedia of north American birds. The Audubon Society, Alfred A. Knopf, New York. Pp. 232-233.,
- Tessier, L., G. Vaillancourt, and L. Pazdernik. 1994. Comparative study of the cadmium and mercury kinetics between the short-lived gastropod *Viviparus georgianus* (Lea) and pelecypod *Elliptio complanata* (lightfoot), under laboratory conditions. Environ. Pollut. 85:271-282.
- Thompson, N.J. 1976. Fat-soluble vitamins. Comp. Anim. Nutr. 1:99-135.
- Thornally, P.J., and M. Vasak. 1985. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta 827:36-44.
- Thunberg, T. U.G. Ahlborg, and H. Johnson. 1979. Vitamin A (retinol) status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch. Toxicol. 42:265-274.
- Tizard, I. 1992. Veterinary immunology, an introduction, 4th ed. W.B. Saunders Co., Philadelphia, PA. 497 pp.
- Trump, B.F., E.M. McDowell, and A.U. Arsilia. 1980. Cellular reaction to injury. In: R.B. Hill, and M.F. LaVia (Eds.). Principles of pathobiology, 3rd ed. Oxford University Press, New York. Pp. 20-111.

- Trust, K.A., A. Fairbrother, and M.J. Hooper. 1994. Effects of 7,12-dimethylbezaanthracene on immune function and mixed-function oxygenase activity in the European starling. *Environ. Toxicol. Chem.* 13:821-830.
- Trust, K.A., M.W. Miller, J.K. Ringelman, and I.M. Orme. 1990. Effects of ingested lead on antibody production in mallards (*Anas platyrhynchos*). *J. Wildl. Dis.* 26:316-322.
- USEPA (United States Environmental Protection Agency). 1975. DDT: a review of scientific and economic aspects of the decision to ban its use as a pesticide. EPA 540/1-75-022. Washington, D.C.
- Vaillant, C., C. LeGuellec, and F. Padkel. 1988. Vitellogenin gene expression in primary culture of male rainbow trout hepatocytes. *Gen. Comp. Endocrin.* 70:284-290.
- Vallee, B.L.. 1991. Introduction to metallothionein. *Methods Enzymol.* 205: 3-8.
- Vasak, M. 1991. Large-scale preparation of metallothionein: biological sources. *Methods Enzymol.* 205:39-41.
- Viarengo, A., M. Pertica, M. Mancinelli, G. Zanicchi, and M. Orunesu. 1980. Rapid induction of copper-binding proteins in the gills of metal-exposed mussels. *Comp. Biochem. Physiol.* 67C:215-218.
- Volgelbein, W.K., J.W. Fournie, P.A. Van Veld, and R.J. Huggett. 1990. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a Creosote-contaminated site. *Cancer Res.* 50:5978-5986.

- Vos, J.G. 1977. Immune suppression as related to toxicology. *CRC Crit. Rev. Toxicol.* 5:67-101.
- Vos, J.G. and J.H. Koeman. 1970. Comparative toxicologic study with polychlorinated biphenyls in chickens with special reference to porphyria, edema formation, liver necrosis, and tissue residues. *Toxicol. Appl. Pharmacol.* 17:656-668.
- Walker, C.H., I. Newton, S.D. Hallam, and M.J.J. Ronis. 1987. Activities and toxicological significance of hepatic microsomal enzymes of the kestrel (*Falco tinnunculus*) and sparrowhawk (*Accipiter nisus*). *Comp. Biochem. Physiol.* 86C:379-382.
- Wallack, J.D. and W.J. Boever. 1983. Diseases of exotic animals, medical and surgical management. W.S. Saunders Co. (eds), Toronto. 463pp.
- Walton, M.J., and C.B. Cowey. 1982. Aspects of intermediary metabolism in salmonid fish. *Comp. Biochem. Physiol.* 73B: 59-79.
- Warinner, J.E., E.S. Mathews, and B.A. Weeks. 1988. Preliminary investigations of the chemiluminescent response in normal and pollutant-exposed fish. *Mar. Environ. Res.* 24:281-284.
- Weeks, B.A., A.S. Keisler, J.E. Warinner, and E.S. Mathews. 1987. Preliminary evaluation of macrophage pinocytosis as a fish health monitor. *Mar. Environ. Res.* 22:205-213.
- Weeks, B.A., D.P. Anderson, A.P. Dufour, A. Fairbrother, A.J. Goven, G.P. Lahvis, and G. Peters. 1992. Immunological biomarkers to assess environmental stress. In: R.J. Hugget, R.A. Kimerle, P.M. Mehrle, and H.L.

- Berman (Eds.). Biomarkers: biochemical, physiological, and histological markers of anthropogenic stress. Lewis Publ., Chelsea, MI. pp. 211-234.
- Wobeser, G.A. and W. Kost. 1992. Starvation, staphylococcosis, and vitamin A deficiency among mallards over-wintering in Saskatchewan. J. Wildl. Dis. 28:215-222.
- Wong, S. M. Fournier, D. Coderre, W. Banska, and K. Krzystyniak. 1992. Environmental immunotoxicology. In: D. Peakall (Ed.). Animal biomarkers as pollution indicators. Chapman & Hall, London. Pp.167-189.
- Woods, J.S., M.A. Bowers, H.A. Davis. 1991. Urinary porphyrin profiles as biomarkers of trace metal exposure and toxicology: Studies on urinary porphyrin excretion patterns in rats during prolonged exposure to methyl mercury. Toxicol. Appl. Pharmacol. 110:464-476.
- World Health Organization. 1987. PCBs, PCDDs and PCDFs: Prevention and control of accidental and environmental exposures. Environmental Health Series, Copenhagen. 227p.
- Wormington, A. and J.H. Leach. 1992. Concentrations of migrant diving ducks at Point Pelee National Park, Ontario, in response to invasion of zebra mussels, *Dreissena polymorpha*. Can. Field-Nat. 106:376-380.
- Zuchelkowski, E.M., R.C. Lantz, and D.E. Hinton. 1986. Skin mucous cell response to acid stress in male and female brown bullhead catfish, *Ictalurus nebulosus* (Le Sueur). Aquat. Toxicol. 8:139-148.

IMAGE EVALUATION TEST TARGET (QA-3)



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