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dissertation entitled

Development of in vitro Assays to Assess the Effects of Polychlorinated Diaromatic Hydrocarbons on Immune Responses of Chinook Salmon (Oncorhynchus tshawytscha)

presented by

George E. Noguchi

has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in Fish. & Wildl. & Envir. Tox.

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Date August 11, 1997

DEVELOPMENT OF *IN VITRO* ASSAYS TO ASSESS THE EFFECTS OF POLYCHLORINIATED DIAROMATIC HYDROCARBONS ON IMMUNE RESPONSES OF CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

By

George Edward Noguchi

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Fisheries and Wildlife and
Program in Environmental Toxicology

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ABSTRACT

DEVELOPMENT OF *IN VITRO* ASSAYS TO ASSESS THE EFFECTS OF POLYCHLORINIATED DIAROMATIC HYDROCARBONS ON IMMUNE RESPONSES OF CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

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George Edward Noguchi

In vitro assays were developed to evaluate the direct effects of polychlorinated diaromatic hydrocarbons (PCDHs) on the chinook salmon immune system. Functional immune responses including lymphocyte proliferation and antibody production were used as endpoints for measuring effects on humoral immunity. Conditions which support mitogen-induced proliferation of chinook salmon lymphocytes, *in vitro*, were defined. Culture medium supplemented with homologous salmon plasma was superior to medium supplemented with either autologous salmon plasma or calf serum for supporting maximal mitogen responsiveness. Soluble constituents in salmon plasma along with the proportion of responsive lymphocyte subpopulations were identified as two factors which may contribute to variability in mitogen responsiveness among individual fish.

The *in vitro* approach was effective for detecting effects of PCDHs on salmon lymphocyte proliferation. The sensitivity of chinook salmon lymphocytes to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was comparable to dioxin-sensitive mouse strains and the potency of 3,3',4,4',5-pentachlorobiphenyl (PCB-126) relative to TCDD was similar to other aryl hydrocarbon receptor (AhR) mediated effects in fish. Concentrations of Aroclor 1254 that were immunosuppressive (45 and 90 uM) were also cytotoxic. Results from the

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in vitro assays approximated *in vivo* effects, when effective concentrations were expressed on a cellular or tissue basis, and suggest that concentrations of PCDHs in Lake Michigan chinook salmon present a low risk of affecting lympho-proliferative responses.

Conditions are also described for the *in vitro* production of TNP-specific antibodies by chinook salmon splenocytes and for the detection of antibody activity by ELISA and the detection antibody affinity by affinity ELISA. Concentrations of TCDD that affected antibody synthesis were comparable to concentrations that suppress lymphocyte proliferation (3 and 30 nM). PCB-126 exhibited immunosuppressive tendencies, although great within-treatment variability diminished the statistical significance of these effects. Non-cytotoxic concentrations of Aroclor 1254 (0.45 - 9 μM) did not depress antibody activity. The suppressive effects of TCDD and PCB-126 on antibody production were accompanied by the elimination of antibodies with high affinity for the TNP antigen. These results suggest B cells expressing high affinity antigen receptor may be particularly susceptible to TCDD and other AhR agonists.

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support

Dedicated to my wife, Laura, and children, Anna and William, for their encouragement, support, and love and to my parents, George and Betty, for their unquestioning support, and appreciation of academic achievement and civic responsibility.

ACKNOWLEDGMENTS

Primary support for this research was provided by the U.S. Fish and Wildlife Service, the National Biological Survey and the U.S. Geological Survey. The author's salary and tuition were provided by a training agreement with the Great Lakes Science Center, Ann Arbor, Michigan, and supported by Drs. Michael Mac and John Gannon. Additional support was provided by the Aquatic Toxicology Laboratory and the Tissue Typing Laboratory at Michigan State University, and the Fish Health Laboratory-Leetown Science Center, Kearneysville, West Virginia.

I am indebted to my academic advisor, Dr. John Giesy for his guidance, support, and encouragement throughout my graduate program at Michigan State. Other members of my graduate committee also provided valuable assistance. Dr. Robert Bull, Department of Medicine, was a valued counselor and provided technical resources at the Tissue Typing Laboratory. Dr. Norbert Kaminski, Department of Pharmacology and Toxicology, provided laboratory resources, technical assistance, and guidance. Dr. Frank D'Itri, Department of Fisheries and Wildlife, provided guidance and helpful review comment on the draft of this dissertation.

Several other individuals provided invaluable assistance during the course of this work. Jeff Allen assisted in fish care and sampling at the Great Lakes Science Center and Carol Edsall provided technical assistance, moral support and guidance. Peggy Bull, Lori

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Culham and John Davis at the Tissue Typing Laboratory, Michigan State University, provided assistance with cell culture techniques, mitogen assays, and ELISA methodology. Dr. Louis King, Biochemistry Department, Michigan State University, assisted with flow cytometry analyses and Drs. Stephen Kaattari, Virginia Institute of Marine Sciences, Gloucester Point, Virginia, and Mary Arkoosh, National Marine Fishery Service, Newport, Oregon, provided valuable reagents (monoclonal antibodies). The chinook salmon used in this research were provided by Jim Copeland, Wolf Lake Hatchery, Michigan Department of Natural Resources, Mattawan, Michigan. The students and staff of the Aquatic Toxicology Laboratory at Michigan State provided technical assistance, moral support and endless hours of good conversation.

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INTRODUCTION

Problem Statement

Recent declines in the chinook salmon, *Oncorhynchus tshawytscha*, population in Lake Michigan have been attributed to massive die-offs of fish infected with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD; Smith, 1992). Although BKD appears to be the ultimate cause of death, other factors are thought to have played a role in rendering salmon susceptible to this otherwise ubiquitous bacteria. Organic contaminants are among the group environmental factors which have been considered by the Lake Michigan Chinook Salmon Mortality Subcommittee as possibly contributing to the salmon mortality. Detectable levels of contaminants including, dioxins and dibenzofurans (Smith et al., 1990) and polychlorinated biphenyls (PCB; Masnado, 1987; Smith et al., 1990; Williams et al., 1992) have been measured in chinook salmon collected from Lake Michigan waters. These polychlorinated diaromatic hydrocarbons (PCDH) are potent mammalian immunotoxins (Vos and Luster, 1989) which reduce host resistance to infection and disease. The efficacy of these compounds to suppress immune responses in fish is less definitive because of the limited number of studies reported and apparent differences in sensitivity among species.

Most laboratory studies evaluating the immunotoxicity of dioxins (Spitsbergen et al., 1986) or PCBs (Cleland et al., 1988a & 1988b; Spitsbergen et al., 1988c; Thuvander et al.,

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1993) have been conducted using rainbow trout, *Oncorhynchus mykiss*, and indicate that immune responses of this species are not greatly affected at sublethal doses. Recent studies, however, suggest that chinook salmon may be more sensitive to this class of chemicals. The primary and secondary humoral immune responses of chinook salmon were significantly reduced in fish injected with PCB at a sublethal dose (54 mg Aroclor 1254 kg^{-1} salmon; Arkoosh et al., 1994a). In addition, sublethal concentrations of PCBs and PAHs (polycyclic aromatic hydrocarbons) present in Puget Sound, an urban estuary, were associated with suppression of the secondary humoral immune response of juvenile salmon inhabiting the area (Arkoosh et al., 1991). Concentrations of PCBs in Lake Michigan chinook salmon range from 1.2 to 4.1 mg kg^{-1} in whole fish (Noguchi, unpublished data) and concentrations in specific tissues range from 1 mg kg^{-1} in muscle (Williams et al., 1992) to as great as 13.6 mg kg^{-1} in gonads (Williams and Giesy, 1992). PCBs accumulated in Lake Michigan salmon are enriched in toxic (planer) PCB congeners such that the toxic potency is estimated to be about 3.3 times greater than Aroclor 1254, a technical PCB mixture (Williams and Giesy, 1992). Therefore, the toxic potency attributable to the dioxin-like compounds in PCB mixtures in Lake Michigan chinook salmon are equivalent to a concentration of Aroclor 1254 that is 3.3 times greater or 4 to 13.5 mg kg^{-1} whole body and up to 44.9 mg kg^{-1} in selected tissues. These concentrations approach the dose (54 mg kg^{-1}) that has been shown to be immunosuppressive and indicate that concentrations of PCBs in Lake Michigan chinook salmon may be high enough to suppress immune functions and potentially decrease the resistance of salmon to disease.

Although concentrations of PCBs in Great Lakes fish have decreased significantly over the past two decades they appear to be stabilizing at concentrations that still exceed

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many state and federal action levels (Cunningham et al., 1990) and are above concentrations estimated to be protective for sensitive wildlife species (Ludwig et al., 1993). Immunological endpoints may help to identify fish species that are at greater risk of infection and disease because of their sensitivity to PCBs and other xenobiotics.

Research Goals

The salient questions that need to be answered are whether PCBs and other PCDHs are immunosuppressive to chinook salmon at concentrations observed in Lake Michigan and if their effects contribute to the susceptibility of salmon to BKD. This is a large task and no single study will completely answer the question. My research was designed to answer some fundamental questions regarding the sensitivity of chinook salmon to PCDHs and allow for more accurate predictions of the immunological consequences of environmental PCDH concentrations. *In vitro* assays were adapted to evaluate the direct effects of PCDHs on target cells of the salmon immune system. Functional responses such as lymphocyte proliferation and antibody production were used as endpoints for measuring immunosuppressive action. The efficacy of non-ortho (dioxin-like) substituted PCB congeners were assessed to determine if structure-activity relationships indicative of an Ah-receptor mediated mechanism could be observed. Cellular concentrations of PCBs were measured to provide tissue-specific doses. As with any *in vitro* approach, effects measured in cell cultures do not necessarily measure whole animal responses. However, *in vitro* tests are critical for identifying sensitive cell-types and responses, and mechanisms of action. These assays could be adapted for assessing effects following *in vivo* exposure to contaminants, for monitoring the immune status of feral salmon and they could also be used

to test effects of weathered complex mixtures of residues extracted from fish tissues.

CHAPTER 1

Literature Review: Immunological Disorders in Fish Associated with Polychlorinated Biphenyls and Related Halogenated Aromatic Hydrocarbon Compounds

(Submitted for publication as a chapter in *Fish Diseases and Disorders, Volume III: Non-Infectious Disorders*, eds: J.F. Leatherland and P.T.K. Woo)

INTRODUCTION

The immune system protects the body from disease by detecting and neutralizing disease-causing pathogens (viruses, bacteria, fungi, and parasites) and transformed (neoplastic) cells. In order for the immune system to be effective it must be capable of discriminating between what is foreign and what is not foreign, *i.e.*, "self". The process of self-nonself discrimination involves intricate interactions between target cells (*e.g.*, pathogens and tumor cells) and both cellular and humoral (soluble) elements of the immune system. Once foreign agents are detected, they are subjected to a vast array of effector cells (phagocytes, granulocytes, cytotoxic cells and natural killer cells) and soluble factors (antibodies, complement) that facilitate neutralizing, killing and clearing of the inducing agent. Disruption or modulation of these interactions by drugs or chemical

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contaminants is the subject of immunotoxicology. Exposure to immunotoxic chemicals may result in a variety of disorders including immunosuppression, immunopotentiaiton, immuodeficiency, hypersensitivity or autoimmuity (Dean and Murray, 1991). Although most of what is known about the action of immunotoxic compounds is based on the mammalian immune system there is increasing interest in assessing effects on lower vertebrates, some of which may accumulate high concentrations of immunomodulating chemicals in the environment.

Fish immunotoxicology is an emerging field of study. Recent reviews (Weeks et al., 1992; Dunier and Siwicki, 1993; Wester et al., 1994; Zelikoff, 1994, Anderson and Zeeman, 1995) and symposia (Stolen and Fletcher, 1994) report on the manner in which immune functions in fish may be modulated by toxic xenobiotic compounds especially mammalian immunotoxins or by pollutants associated with contaminated habitats where fish health is impaired. However, compared to mammalian immunotoxicology, where efforts have been focused on relatively few, well characterized and extensively investigated animal models, much less is known about the effects in fish. This is due, in part, to the large number of fish species studied, the lack of many fish-specific reagents (e.g., monoclonal antibodies that detect cell-surface markers on fish leucocytes and secretory products), and fewer researchers in the field. Nevertheless, there are published reports describing lesions in lymphoid tissues, altered immune functions, or increased disease susceptibility in toxicant-exposed fish or in fish collected from contaminated areas (Tables 1 & 2). This review characterizes immunological disorders in fish associated with the widespread environmental contaminant, polychlorinated biphenyls (PCBs), and related halogenated aromatic hydrocarbons (HAHs). Special attention

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is devoted to comparing the sensitivity of fish species, identifying sensitive immunological endpoints and postulating mechanisms of action.

TOXICITY OF HALOGENATED AROMATIC HYDROCARBONS (HAHs)

Halogenated aromatic hydrocarbons comprise a class of chemicals that induce pleiotropic effects in mammals including immunomodulation (Vos and Luster, 1989). Polychlorinated dibenzofurans, polychlorinated dibenzo-p-dioxins (dioxins) and PCBs are among the most toxic HAHs (Figure 1) and are also ubiquitous environmental contaminants. Because of their resistance to degradation and high lipophilicity, HAHs tend to be biomagnified in aquatic food chains. As a result, detectable concentrations of HAHs have been measured in fish throughout the North America (Smith et al., 1990). The most toxic member is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Several fish species are very sensitive to the lethal effects of TCDD (LD_{50} 3-16 $\mu g\ kg^{-1}$; Kleeman et al., 1988), particularly when compared to sensitive mammalian species. In fact, the early life stages of salmonids are most sensitive to TCDD (LD_{50} 0.065 - 0.230 $\mu g\ kg^{-1}$; Walker et al., 1991; Walker and Peterson, 1991).

The mechanism by which TCDD exerts many of its toxic and biochemical effects is believed to require binding to the cytosolic aryl hydrocarbon receptor (AhR; Poland and Knutson, 1982). Among the sublethal effects associated with AhR binding is the induction of cytochrome P4501A1, a mixed function oxygenase responsible for HAH metabolism. PCB congeners that are structurally similar to TCDD, in that they can attain a planar configuration and are chlorinated in meta and para positions, also bind the AhR and induce P4501A1 activity. Of the 209 PCB congeners, relatively few have high affinity for the AhR (Safe,

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1987). In fish only, nonortho-substituted tetrachloro (3,3',4,4'-tetrachloro biphenyl, 3,3',4,5'-tetrachloro biphenyl), pentachloro (3,3',4,4',5-pentachloro biphenyl) and hexachlor (3,3',4,4',5,5'-hexachloro biphenyl) congeners are known to induce AhR-mediated responses (Janz and Metcalfe et al., 1991; Walker et al., 1991; Newsted et al., 1995). AhR-active PCB congeners are a small percentage of the total mass of commercial PCB formulations such as Aroclor® 1254, which contains over 50 different congeners (Ballschmiter and Zell, 1980). Thus, compared to TCDD, greater doses of commercial PCB mixtures are required to produce similar effects (*e.g.*, chinook salmon, *Oncorhynchus tshawystscha*, LD_{50}: 270 mg Aroclor® 1254 kg^{-1}; Arkoosh et al., 1994a).

OVERVIEW OF THE TELEOST IMMUNE SYSTEM

The detection of sublethal effects of PCBs and other HAHs on the fish immune system has evolved along with the fundamental understanding of immunological processes in fish. The teleost immune system including non-specific and specific immunity, humoral or antibody producing and cell-mediated responses, is shown in Figure 2. The piscine immune system as it relates to protective immunity (innate and acquired) is comprehensively reviewed earlier (van Muiswinkel, 1995; Iwama and Nakanishi, 1996). The intent is to provide a framework with which to consider the implications of immunotoxic effects and not to describe in great detail all aspects of the fish immune system.

Phagocytic cells analogous to mammalian monocytes, macrophages and neutrophils (Ellis, 1977; Fänge, 1992) confer nonspecific immunity by detecting, engulfing, killing and clearing pathogens. Phagocytes serve as both the first line of defense against infection and as effector cells in the humoral immune response. Natural cytotoxic cells (NCC) detect and

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lyse transformed target cells and protozoan parasites (Evans and Jaso-Friedmann, 1992). Like their mammalian counterpart, natural killer cells (NK), NCC induce death in target cells by necrotic and apoptotic mechanisms (Greenlee et al., 1991) and are believed to play an important role in the surveillance of tumor cells. Antigen-presenting cells (APC) are phagocytic cells, typically macrophages, that internalize and process antigen and present processed antigen to T-cells (Vallejo et al., 1992). This results in T-cell activation. The existence of T-cells in fish has been based on functional criteria including responses to mammalian T-cell mitogens (Sizemore et al., 1984; Tillitt et al., 1988), mixed lymphocyte reactions (Kaattari and Holland, 1990), and delayed type hypersensitivity reactions (Stevenson and Raymond, 1990); mammalian T-cells are identified by the presence of specific T-cell receptors. Such receptors have yet to be characterized for fish lymphocytes (Chilmonczyk, 1992; Manning and Nakanishi, 1996). T-cells along with macrophages function as accessory cells in the humoral immune response by secreting soluble factors, such as interleukins (ILs), that are required for B-cell activation, proliferation and differentiation (Kaattari, 1992). B lymphocytes express antigen receptors, *i.e.* membrane immunoglobulins (DeLuca et al., 1983), and are capable of binding free (non-processed) antigen. Some polymeric antigens and mitogens can activate B-cells without the participation of T-cells, and are referred to as thymus independent or T-I antigens. Antigens that require T-cell involvement to activate B-cells are termed thymus dependent antigens, T-D. B-cells activated by T-D antigens proliferate and differentiate into either plasma cells, which produce and secrete antibodies, or memory B-cells. Antibodies circulate in the blood stream and bind to specific antigenic features (epitopes) on pathogens that activated the B-cells. These antibody coated (opsonized) pathogens are targeted for deletion by phagocytic cells (macrophages) or

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destroyed by complement-mediated cell lysis (Saki, 1992). Memory B-cells do not participate in the initial or priming exposure to antigen but respond to secondary and subsequent encounters with the specific antigen (Arkoosh and Kaattari, 1991). Secondary humoral responses to antigen occur more rapidly and with greater intensity (more antibody producing cells and higher antibody titers) than the primary response (Arkoosh et al., 1991).

The major lymphoid tissues in teleosts include the anterior kidney (pronephros), thymus and spleen. The anterior kidney is the principal hemopoietic tissue and also functions as a primary lymphoid tissue for B-cell maturation (Kaattari, 1992). The thymus is the primary lymphoid tissue in mammals where T-cell differentiation and maturation occurs. In fish, the thymus is believed to play a similar role, although the precise function is not as well understood (Chilmonczyk, 1992). Mature T and B lymphocytes migrate from primary lymphoid tissues into the blood stream and concentrate in secondary lymphoid tissues (*e.g.*, spleen). The spleen contains high numbers of lymphocytes and macrophages and it also functions as a filter to trap antigens and allow maximal contact between antigen and immunoreactive cells.

EFFECTS OF HAHS ON HUMORAL IMMUNITY

Humoral immune responses, particularly the antibody-forming cell response (AFC), are among the most sensitive indicators of HAH immunotoxicity in higher vertebrates (Davis and Safe, 1988; Vos et al., 1989; Kerkvliet and Burleson, 1994). The AFC response is a measure of the number of antibody forming cells (plasma cells) that are produced in response to immunization with antigen and, therefore, represents an integrated measure of B-cell and accessory cell (macrophage and T-cell) function. The degree to which HAHs affect humoral

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responses appears to be influenced by many factors which include fish species, type of antigen (T-D or T-I) and mode of immunization (*in vivo* or *in vitro*).

Primary humoral responses to T-I antigens are more affected by HAHs than primary responses to T-D antigens (Table 1). In rainbow trout, *Oncorhynchus mykiss*, PCB treatment (500 mg Clophen® A50 kg^{-1} diet) significantly reduced the humoral response (antibody titer) to *Vibrio anguillarum* O antigen, a T-I antigen (Thuvander et al., 1991). Whereas, humoral responses in trout to T-D antigens were not affected by PCBs (Cleland et al., 1988a; Thuvander et al., 1993) or TCDD (Spitsbergen et al., 1986). Similarly, the primary *in vitro* AFC response to a T-I antigen (TNP-LPS), but not a T-D antigen (TNP-KLH), was depressed in juvenile chinook salmon receiving a single dose of Aroclor® 1254 (54 mg kg^{-1}; Arkoosh et al., 1994a). Low doses of PCB 126 (0.01 mg kg^{-1}) actually enhanced the AFC response in channel catfish, *Ictalurus punctatus*, to a T-D antigen, yet, the response was not significantly affected by higher doses (0.1 and 1 mg kg^{-1}; Rice and Schlenk, 1995). The differential effect of HAHs on humoral responses to T-D and T-I antigens in fish may reflect differences in the sensitivity of lymphocyte subpopulations. A discussion of the cellular targets of HAH-induced immunotoxicity is in a later section.

The effect of HAHs on B-cell mediated immunity in chinook salmon indicates that secondary or amnestic responses may be more sensitive than the primary response. The primary *in vitro* AFC response of juvenile salmon to TNP-KLH (a T-D antigen) was not affected by PCB treatment (54 mg Aroclor® 1254 kg^{-1}), however, the secondary response was reduced by more than 90% compared to untreated controls (Arkoosh et al., 1994a). Because this effect occurred at a dose that was less than half of the ED_{50} (118 mg Aroclor® 1254 kg^{-1}) for HAH-sensitive mice (C57BL/6; Davis and Safe, 1989) it would appear that

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chinook salmon is one of the more sensitive species in terms of PCB-induced immunosuppression.

EFFECTS ON NON-SPECIFIC AND CELLULAR IMMUNITY

Although relatively few studies on the immunotoxicity of HAHs included non-specific and cellular immunity, there is evidence which suggests species-specific differences in the sensitivity to these compounds (Table 1). The phagocytic activity of peritoneal macrophages is a measure of non-specific immunity and this was not affected in rainbow trout treated with a lethal dose of TCDD (10 μg kg^{-1}; Spitsbergen et al., 1986). However, the oxidative burst activity in stimulated phagocytes, another indicator of immune competence, was significantly reduced in channel catfish treated with sublethal amounts of PCB 126 (0.1-1.0 mg kg^{-1}; Rice and Schlenk, 1995). In the same study, the activity of natural cytotoxic cells (NCC) was also suppressed in PCB 126-exposed catfish (1.0 mg kg^{-1}). In contrast, NCC activity was not inhibited in rainbow trout receiving prolonged dietary exposure to Aroclor® 1254 (3-300 mg kg^{-1}; Cleland and Sonstegard, 1987). Although these studies examined the effects of different HAHs it would appear that the non-specific and cellular immune responses in rainbow trout are more resistant to HAHs compared to channel catfish.

The proliferative response of lymphocytes to T-cell mitogens is another measure of cellular immunity. Neither TCDD (Spitsbergen et al., 1986) nor Clophen® A50 (Thuvander et al., 1993) significantly affect the response of rainbow trout lymphocytes to T-cell mitogens. However, in rainbow trout previously immunized with KLH, the responses to both phytohaemagglutinin (PHA; a mammalian T-cell mitogen) and lipopolysaccharide (LPS; a mammalian B-cell mitogen) were significantly enhanced following exposure to Clophen® A50

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(80 mg/kg; Thuvander et al., 1993). These results suggest that HAHs differentially affect lymphocyte activity and it depends on the immune status of the fish prior to chemical exposure. Enhanced mitogen responsiveness was also observed by Faisal et al. (1991a) in contaminant-exposed spot, *Leiostomus xanthurus*. The authors suggested that greater LPS responsiveness of spot leucocytes may have been due to contaminant-induced inhibition of suppressor T-cell activity. [PCBs have been shown to decrease T suppressor activity of murine leucocytes (Kerkvliet and Baecher-Steppan, 1988).] Lymphocytes with T suppressor activity are believed to participate in the regulation of immune functions in fish (Kaattari et al., 1986), however, the role of suppressor T cells in mediating HAH-induced immunomodulation has not been fully explored.

PATHOLOGY OF LYMPHOID TISSUES

Thymic involution, or reduction in size and cellularity of the thymus, is an indication of TCDD toxicity in mammals (Vos et al., 1989). TCDD-induced lesions in the lymphoid tissues of fish have been detected but they usually occur at lethal or near-lethal doses (Table 2). Thymic lesions, characterized by multiple invaginations of the thymic epithelium extending into a lymphoid-depleted cortex, were described by Spitsbergen et al. (1988a) in rainbow trout receiving a lethal dose of TCDD (10 μg TCDD kg^{-1}; the 80-d LD_{50}). These fish also exhibited splenic lymphoid depletion as well as depletion of lymphomyloid elements in the pronephros and midkidney. No lesions were found in trout dosed with sublethal amounts of TCDD. Splenic lymphoid depletion was detected by van der Weiden et al. (1992) in rainbow trout dosed with lower levels of TCDD (0.6 and 3.06 μg TCDD kg^{-1}). These doses were near or below the lethal threshold (20% mortality at 3.06 μg TCDD kg^{-1}) and in the

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range where moderate hepatic EROD activity (EC_{50} 0.79 μg TCDD kg^{-1}) was induced. Differences in the sensitivity of various rainbow trout strains to TCDD have been reported for other toxicological endpoints (early life stage mortality; Walker and Peterson, 1991) and could have contributed to the differences in sensitivity. Percids are also sensitive to TCDD. Mild to moderate splenic lymphoid depletion in yellow perch, *Perca flavescens*, occurred at lower doses of TCDD (5 μg kg^{-1}) than thymic involution and pronephric lymphoid depletion (>25 μg TCDD kg^{-1}; Spitsbergen et al., 1988b), however, these lesions were not detected at doses below the 80-d LD_{50} (3 μg TCDD kg^{-1}).

In studies in which fish were exposed to PCBs, lesions in thymic and/or splenic tissues were not always observed. Splenic lesions were found in rainbow trout exposed to dietary levels of PCBs ranging from 10 to 500 mg Aroclor® 1254 kg^{-1} (Nestel and Budd, 1975; Hendricks et al., 1977; Spitsbergen et al., 1988c). These levels were not reported to be lethal over the course of these studies (75 days to 12 months). Thymic and splenic hypocellularity were noted in rainbow trout injected with a sublethal dose (80 mg/kg) of Clophen® A50 (Thuvander et al., 1993). However, no lesions were detected in rainbow trout fed 500 mg Clophen® A50 kg^{-1} for 10 weeks; although significant effects on humoral immunity were observed (Thuvander et al., 1991). Similarly, humoral immune responses were suppressed in chinook salmon injected with 54 mg Aroclor® 1254 kg^{-1}, but no lesions in lymphoid tissues were detected (Arkoosh et al., 1994a). Thus, lesions in lymphoid tissues are not always associated with HAH exposure or HAH-induced effects on immune functions.

EFFECTS ON DISEASE RESISTANCE

The ultimate manifestation of immunotoxicity is the ability of a toxicant to increase

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disease susceptibility. However, relatively little is know about the effects of HAHs on disease resistance in teleosts other than in rainbow trout. In this species, disease resistance has not been compromised by exposure to HAHs. Neither median time to death (MTD) nor cumulative mortality in rainbow trout challenged with infectious hemopoietic necrosis virus (IHNV) was affected by exposure to TCDD (0.01 - 1 μg kg^{-1} body weight) or PCB (5 - 500 mg Aroclor® 1254 kg^{-1} diet; Spitsbergen et al., 1988c). However, lesions characteristic of IHNV-induced disease were more severe in fish treated with PCBs or TCDD, which indicates that HAHs may enhance progression of the disease without hastening mortality. Similarly, MTD in rainbow trout challenged with *Yersinia ruckeri* was not shortened following 90-d water-borne exposure to PCBs (0.23 - 2.9 ug Aroclor® 1254:1260 L^{-1}; Mayer et al., 1985). In addition, resistance of rainbow trout to *Vibrio anguillarum* was not compromised in fish fed HAH-contaminated diets consisting of Pacific or Great Lakes coho salmon (0.02 - 2.3 μg PCB g^{-1}; Cleland et al., 1988b). These findings are consistent with the relative ineffectiveness of HAHs at altering humoral and cellular immunity in this species. However, impaired disease resistance associated with HAH exposure has been reported for other fish species. Immunization against *Aeromonas hydrophila* was ineffective at protecting PCB-treated (70 mg Aroclor® 1232/kg) channel catfish from a challenge with the virulent bacterium (Jones et al., 1979). More recently, Arkoosh et al. (1994a) reported that juvenile chinook salmon retrieved from an urban estuary contaminated with PCBs and polycyclic aromatic hydrocarbons (PAHs) suffered higher mortality following exposure to *Vibrio anguillarum* than salmon from a noncontaminanted estuary or salmon held in a hatchery. The humoral immune response was depressed in salmon form the same contaminated estuary (Arkoosh et al., 1991).

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FIELD OBSERVATIONS

Establishing cause-effect relationships between a suspected chemical agent and effects observed in wild fish populations (*i.e.*, epizootiology) can be complicated by uncontrollable factors that may potentiate, or mask or independently induce the effect(s). Nevertheless, detection of strong associations between chemical contaminants and biological effects can strengthen the argument for causality when the same effects have been demonstrated in controlled laboratory studies.

Altered immune functions have been detected in feral fish from field locations known to be contaminated with HAHs and other organic and inorganic contaminants. Carlson and Bodammer (1994) found that the humoral immunity was compromised in winter flounder, *Pleuronectes americanus*, inhabiting an area of Long Island Sound (Morris Cove - New Haven Harbor) that was contaminated with PCBs, PAHs and heavy metals. The authors measured the *in vitro* AFC response of splenic lymphocytes and observed that the response to both T-I (TNP-LPS) and T-D (TNP-KLH) antigens in fish from the Morris Cove site was about 50% lower than the response in fish from a less contaminated reference site. Humoral immunity was also depressed in juvenile chinook salmon that were collected from an HAH-PAH contaminated urban estuary in Puget Sound (Arkoosh et al., 1991). Although no effects were observed in the primary response, the secondary *in vitro* AFC response of anterior kidney leukocytes in salmon from the contaminated urban estuary was significantly less than the response in hatchery salmon or in salmon collected from a non-urban estuary. Several reports have also documented altered immune functions in fish from sections of the Elizabeth River (Virginia) that are heavily contaminated, primarily with PAHs but also with HAHs (Huggett et al., 1992). The immunological disorders in fish from that system include,

diminished natural cytotoxic cell activity (Faisal et al., 1991b), reduced phagocytic and chemotactic activity of kidney macrophages (Weeks et al., 1990), and altered responsiveness of pronephric lymphocytes to mitogenic stimulation (Faisal et al., 1991a). The abundance of macrophage aggregates in wild fish has been positively correlated with concentrations of HAHs and other contaminants in bottom sediments (Blazer et al., 1994). Macrophage aggregates are accumulations of pigmented macrophages, in the spleen, kidney and liver with normal physiological and immunological functions (Wolke, 1992). Changes in abundance of macrophage aggregates may be due to contaminant-induced stress. Establishing causal relations between immunological disorders and environmental exposure to HAHs requires an understanding of the mechanisms by which HAHs modulate the immune system.

MECHANISMS OF IMMUNOMODULATION

Many of the pleiotropic effects attributed to HAHs are mediated by a process that requires initial binding of ligand to the AhR (Figure 3). Support for the essential role of AhR-ligand binding is based primarily on two lines of evidence 1) quantitative structure-activity relationships between AhR binding affinity and toxic potency and 2) the differential sensitivity of mouse strains possessing alleles encoding high and low affinity AhR. TCDD is the prototypical AhR agonist. The AhR binding affinity of other HAHs is greatest for planer congeners that are structurally most similar to TCDD (Poland et al., 1976; Safe et al., 1986). Toxic responses (weight loss, thymic atrophy, and immunomodulation) and biochemical responses (enzyme induction) to HAHs are correlated with AhR binding affinity (Poland et al., 1976; Safe, 1987; Davis and Safe, 1988; Kerkvliet et al., 1990a). Thus, TCDD-like toxicity is observed with HAH congeners that bind with high affinity to the AhR. Similarly,

mouse strains possessing the AhR allele that expresses a receptor with high TCDD binding affinity are much more sensitive to biologically active HAHs than mouse strains that express receptor with low binding affinity (Silkworth and Gaberstein, 1982; Vecchi et al., 1983; Tucker et al., 1986; Birnbaum et al., 1990; Kerkvliet et al., 1990b). The TCDD binding affinity of AhR in responsive mouse strains (C57BL/6J mice) is 10 fold greater than in DBA/2J mice, a low responsive strain (Okey et al., 1989). Binding of HAHs to the AhR is a prerequisite for many physiological and biochemical effects.

The most well studied of the TCDD-related effects is the induction of cytochrome P450IA1 (a mixed function oxygenase) that is encoded by the CYPIA1 gene (Figure 3). P450IA1 induction requires initial binding of ligand (TCDD or other active HAH congeners) to the AhR followed by a transformation of the receptor and translocation of the ligated AhR to the nucleus and binding with ARNT, the aryl hydrocarbon nuclear translocator protein (Nebert and Jones, 1989; Whitlock, 1990; Hankinson, 1995). In the nucleus, the AhR-ligand heterodimer binds to dioxin responsive enhancer (DRE) regions in the 5' flanking region of the CYPIA1 gene. Binding of the AhR-ligand complex to DREs enhances transcription of the downstream gene, CYPIA1. Thus, the DRE binding form of the AhR-ligand complex functions as a transcription factor for CYPIA1, resulting in elevated CYPIA1 transcription and increased levels of the P450IA1 protein.

Induction of detoxification enzymes such as P450IA1 is an adaptive response and not necessarily a measure of toxicity. Whether TCDD acts through this same mechanism to induce toxic responses has yet to be demonstrated unequivocally. However, CYPIA1 is not the only gene that is responsive to the AhR. Sutter and Greenlee (1992) have classified a number of genes that belong to the Ah gene battery. Members of this family include growth

factors (interlukin-1 and transforming growth factor-α) and intracellular proteins involved in signal transduction (phospholipase A2, protein kinase C and tyrosine kinases). It is possible that some of the TCDD-related toxic effects may involve direct interactions with DREs that regulate the transcription of growth factors or other regulators of cellular activity. There is also evidence that the AhR-ligand complex can modulate the phosphorylation of cytosolic proteins that are involved in signal transduction pathways (Puga et al., 1992; Matsumura, 1994). Such alterations could affect the responsiveness of cells to extracellular stimuli.

Recent studies by Masten and Shiverick (1995) suggest that the suppressive effect of TCDD on B-lymphocyte activation and antibody production may involve a direct effect of the TCDD-AhR complex on gene expression. CD19 is a membrane receptor expressed on the surface of mammalian B lymphocytes and participates in B-cell activation and differentiation (Kehrl et al., 1994; Tedder et al., 1994). Treatment of a human B-lymphocyte cell line (IM-9) with TCDD resulted in a 67% decrease in CD19 mRNA, indicating that TCDD may affect CD19 gene expression. The promoter region for the CD19 gene contains a binding site for BSAP, the B cell lineage-specific activator protein (Kozmik et al., 1992). BSAP regulates CD19 gene expression and is believed to play a role in early neurological development as well (Urbanek et al., 1994). The DNA binding site for BSAP contains a five base sequence identical to the DRE consensus sequence. These results suggest that binding sites for the AhR-ligand complex exist in regulatory regions for genes that modulated B-cell activation and differentiation. In the case of CD19, the AhR-ligand complex may compete with the endogenous ligand (BSAP) for binding to the BSAP binding site, resulting in reduced CD19 transcription. Fewer CD19 transcripts may result in reduced expression of CD19 on the cell surface and a diminished capacity to bind and respond to extracellular stimulation. Thus, the

DNA binding activity of the TCDD-AhR complex may not only act to "turn genes on" but may also interfere or compete with other transcription factors thereby reducing gene expression and altering cellular functions

Despite the substantial body of evidence supporting AhR involvement in numerous HAH-induced effects there are some notable exceptions which indicate that HAHs may act through other mechanisms. One particular dioxin congener which lacks AhR binding affinity, 2,7-dichlorodibenzo-p-dioxin (Poland and Glover, 1976), suppresses the AFC response of mouse splenocytes both *in vivo* (Holsapple et al., 1986a) and *in vitro* (Holsapple et al., 1986b). Unlike TCDD, the immunosuppressive effects of 2,7-dichlorodibenzo-p-dioxin are not accompanied by elevated levels of hepatic P4501A1. Other dichlorinated dioxin congeners that have low AhR binding affinity, such as 2,8-dichlorodibenzo-p-dioxin, do not suppress the AFC response (Tucker et al., 1986). Thus, 2,7-dichlorodibenzo-p-dioxin appears to act through a unique mechanism which does not require AhR binding in order to suppress B-cell immunity.

Results from studies with high and low AhR responsive mouse strains also suggest that some immunosuppressive effects of HAHs may be mediated by AhR-independent mechanisms. As previously mentioned, the immunosuppressive effects of HAHs have been shown segregate with the AhR alleles. However, Morris and co-workers (1992) have demonstrated that the exposure regime can greatly influence the responsiveness of low AhR-responsive mice. DBA/2 mice that received subchronic doses of TCDD exhibited a 10-fold enhancement in humoral immune suppression compared to DBA/2 mice that received the same cumulative dose of TCDD but in an acute exposure. In addition, the severity of immunosuppression in subchronically exposed DBA/2 mice was comparable to the

suppression observed in B6C3F1 (AhR responsive) mice. These findings are supported by results from *in vitro* exposures in which TCDD was equally effective at suppressing the AFC response in splenocytes from both high and low AhR responsive mouse strains (Holsapple et al., 1986b). The mechanism by which HAHs induce immunotoxic effects, independent of the AhR, is believed to involve modulation of intracellular Ca^{2+} (Holsapple et al., 1991a). Taken together, these findings indicate that several factors can modulate the immunosuppressive activity of HAHs and that AhR involvement may be critical for many but not all toxic responses.

The role of the AhR in HAH-induced immunodepression in fish is not well understood. Appreciable amounts of AhR have only recently been detected in fish cells (20 fmol/mg protein; Lorenzen and Okey, 1990). However, cytochrome P4501A1 induction has been measured in fish liver, kidney, and gill (Miller et al., 1989; Goksoyr and Förlin, 1992). Structure-activity relationships in fish for P4501A1 induction (Janz and Metcalfe, 1991; Newsted et al., 1995) and early life stage mortality (Walker and Peterson, 1991) suggest that these effects are mediated through the AhR. Although AhR agonists have been shown to affect various immune responses in fish, as discussed previously, there is insufficient information at present to determine whether these effects on dependent on AhR mediated processes.

Several approaches have been used to identify cellular targets in HAH-induced immunotoxicity. Results from *in vitro* and *ex vivo* recombination studies with inbred mice indicate that suppression of the AFC response by TCDD is due to an alteration in the function of B-cells, and not T-cells nor antigen presenting cells (Dooley and Holsapple, 1988). TCDD has been shown to directly affect B-lymphocyte differentiation under *in vitro* conditions

(Tucker et al., 1986; Luster et al., 1988). However, T-cells appear to be more sensitive than B-cells when the effects of dioxins on the AFC response are tested *in vivo* (Kerkvliet and Brauner, 1987). This conclusion is based on the finding that mice immunized with T-D antigens are more sensitive to the suppressive effects of dioxin (1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin; HpCDD) than mice immunized with T-I antigens. Because the AFC response to T-D antigens requires greater T-cell involvement, the logical explanation for the antigen-dependent sensitivity to HpCDD is impaired T-cell function. It is not clear why differences in dosing and immunization schemes would result in differential sensitivity of B and T-cells, although Kerkvliet and Burleson (1994) suggested that dioxins might affect activated T-cells, *in vivo*, through indirect mechanisms. Indirect effects are known. Depletion of thymocytes associated with TCDD-induced thymic atrophy is believed to occur indirectly through cell-cell contact with TCDD-affected thymic epithelial cells (Greenlee et al., 1985).

In fish, it seems B-cells are a target of HAH-induced depression of the primary AFC response because significant effects have been demonstrated with T-I antigens. Surprisingly, responses to T-D antigens are less affected. Perhaps stimulation provided by T lymphocytes in some way protects fish B-cells from the modulatory effects of HAHs. If this is so, then the activation of naive T-cells would have to be less affected by HAHs. Lower T-cell sensitivity may be inferred from the study by Spitsbergen et al. (1986). TCDD treatment depressed the proliferative response of rainbow trout splenocytes to poke weed mitogen, a stimulator of B and T lymphocytes, but did not significantly affect the response to Con A (a mammalian T-cell mitogen).

The heightened sensitivity of the secondary AFC response to T-D antigens observed in PCB-treated chinook salmon, indicates that T-cell mediated events may be affected in the memory response. Arkoosh et al. (1994a) suggest that if fish have a requirement for memory T-cells similar to that of mammals then PCBs may affect the transition of naive T-cells to memory T-cells. Such an effect would reduce the pool of memory cells available to participate in the secondary AFC response. Further progress in identifying the mechanisms of HAH immunotoxicity will undoubtedly require both *in vivo* and *in vitro* approaches given the complexity of immune responses and the multiplicity of HAH-associated effects.

CONCLUSIONS

HAHs can disrupt normal immune functions in fish, but some species are more severely affected than others. For example, rainbow trout, one of the more thoroughly studied species, seems to be less sensitive than chinook salmon or channel catfish. Humoral immunity, particularly the secondary AFC response, is one of the more sensitive indicators of HAH immunotoxicity. Nonspecific and cell-mediated responses have not been as thoroughly investigated, although some effects have been reported. Histological lesions in lymphoid tissues, similar to those described in mammals, have been observed in HAH-treated fish but the incidence and severity of these lesions has not always coincided with impaired immune function. Immunodepression has been reported in wild fish inhabiting areas contaminated with HAHs and other organic and inorganic pollutants. However, a better understanding of the mechanisms underlying HAH-induced immunomodulation and of the sensitivity of fish species in aquatic communities will be required to more accurately assess the risk posed by environmental exposure to HAHs.

FUTURE CONSIDERATIONS

One of the major limitations in identifying sensitive immunological endpoints of HAH immunotoxicity has been the fish-to-fish variability often encountered in measuring immune responses. In some studies the coefficient of variation (a measure of within-group variability) has far exceeded 50% (Spitsbergen et al., 1986; Thuvander et al., 1993). This tremendous variation increases the probability of type II error (*i.e.*, accepting the null hypothesis when in fact there were real differences). Mammalian immunotoxicologists have the advantage of working with inbred and syngeneic strains of animals that respond more consistently. This permits greater sensitivity in detecting subtle differences. Inbred fish strains are being developed (Komen et al., 1990) and this will improve the sensitivity of these studies. Alternatively, *in vitro* techniques using tissue sections (Anderson, 1992) or primary cell cultures (Noguchi et al., 1994, 1996) from an individual fish will allow the effects to be measured in genetically identical cell populations. *In vitro* approaches are valuable for studying mechanisms of action, assessing the intrinsic sensitivity of individual fish, and to help identify factors that may account for variability in immune responses between fish.

HAHs and other contaminants represent only one of the many environmental factors that may affect the immune status of wild fish. Identification of HAH-specific immunological perturbations (perhaps effects on the secondary AFC response) may help to distinguish chemical-induced effects from other contributing factors such as nutrition (Blazer, 1992), temperature (Clem et al., 1992), or season (Zapata et al., 1992). Currently, it is necessary to employ a battery of immunological and other tests (*e.g.* enzyme induction) to generate a profile of immunomodulation that characterizes a chemical etiology.

Table 1. Laboratory studies investigating the effects of HAHs on functional immune responses in fish. Effects are indicated by following symbols: (-) no statistically significant difference between chemically treated and non-treated fish, (↓) significant decrease in response, (↑) significant increase in response.

Species	Response	Chemical (dose & route)	Effect	Endpoint - Antigen	Reference
Chinook Salmon	Humoral	Aroclor ® 1254 54 mg kg^{-1} IP	- ↓	Primary *in vitro* AFC - TNP-KLH (T-D) Primary *in vitro* AFC - TNP-LPS (T-I)	Arkoosh et al. (1994a)
Chinook Salmon	Humoral	Aroclor ® 1254 54 mg kg^{-1} IP	↓ ↓	Secondary *in vitro* AFC - TNP-KLH (T-D) Secondary *in vitro* AFC - TNP-LPS (T-I)	Arkoosh et al. (1994a)
Rainbow Trout	Humoral	Aroclor ® 1254 3,30,300 mg kg^{-1} diet	-	AFC - Sheep red blood cells (T-D)	Cleland et al. (1988a)
Rainbow Trout	Humoral	Chlophen ® A50 500 mg kg^{-1} diet	↓	Antibody titer - V. anguillarum O antigen (T-I)	Thuvander et al. (1991)
Rainbow Trout	Humoral	Chlophen ® A50 40 & 80 mg kg^{-1} IP	-	Antibody titer - KLH (T-D)	Thuvander et al. (1993)
Rainbow Trout	Humoral	Chlophen A50 80 mg kg $®^{-1}$ IP	- ↑	Proliferation - LPS Proliferation - LPS (in fish previously immunized with KLH)	Thuvander et al. (1993)
Rainbow Trout	Humoral	TCDD .1, 1, 10 µg kg^{-1} IP	- -	Antibody titer - Sheep red blood cells (T-D) AFC - Sheep red blood cells (T-D)	Spitsbergen et al. (1986)
Rainbow Trout	Humoral	TCDD 10 µg kg^{-1} IP	↓	Proliferation - Poke weed mitogen	Spitsbergen et al. (1986)
Channel Catfish	Humoral	PCB 126 .01 mg kg^{-1} IP 0.1 & 1 mg kg^{-1} IP	↑ -	AFC - Edwardsiella ictaluri (T-D)	Rice and Schlenk (1995)

Table 1 (continued).

Species	Response	Chemical (dose & route)	Effect	Endpoint - Antigen	Reference
Rainbow Trout	Cellular	TCDD .1, 1, 10 μg kg^{-1} IP	-	Proliferation - Conconavelin A	Spitsbergen et al. 1986
Rainbow Trout	Cellular	Chlophen ® A50 80 mg kg^{-1} IP	- ↑	Proliferation - PHA Proliferation - PHA(in fish previously immunized with KLH)	Thuvander et al. (1993)
Channel Catfish	Non-Specific	PCB 126 .1 &1 mg kg^{-1} IP	↓	Oxidative Burst	Rice and Schlenk (1995)
Rainbow Trout	Non-Specific	TCDD 10 μg kg^{-1} IP	-	Phagocytosis	Spitsbergen et al. (1986)
Channel Catfish	Non-Specific	PCB 126 1 mg kg^{-1} IP	↓	NCC Activity	Rice and Schlenk (1995)
Rainbow Trout	Non-Specific	Aroclor ® 1254 3-300 mg kg^{-1} diet	-	NCC Activity	Cleland and Sonstegard (1987)

Table 2. Pathology of lymphoid tissues from fish exposed to HAHs.

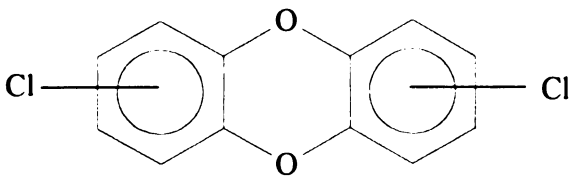
Species	Chemical (dose & route)	Tissue - Pathology	Reference
Rainbow Trout	TCDD 0.6 & 3.06 μg kg^{-1} IP	Spleen - lymphoid depletion and hyperemia (congestion of erythrocytes)	van der Weiden et al. (1992)
Rainbow Trout	TCDD 1μg kg^{-1} IP 10 μg kg^{-1} IP	No lesions in thymus, spleen or kidney Thymus - multiple invagtinations, lymphoid-depleted cortex Spleen - lymphoid depletion Kidney - depletion of lymphomyloid elements	Spitsbergen et al. (1988a)
Yellow Perch	TCDD 5 μg kg^{-1} IP 25 & 125 μg kg^{-1} IP	Spleen - mild to moderate lymphoid depletion Spleen - severe lymphoid depletion Thymus - thymic involution Kidney - moderate depletion of lymphoid and hematopoietic elements	Spitsbergen et al. (1988b)
Rainbow Trout	Aroclor ® 1254 10 & 100 mg kg^{-1} diet	Spleen - reduced amount of white pulp (lymphoid elements)	Nestel and Budd (1975)
Rainbow Trout	Aroclor ® 1254 100 mg kg^{-1} diet	Spleen - reduced amount of white pulp and hyperemia	Hedricks et al. (1977)
Rainbow Trout	Aroclor ® 1254 50 & 500 mg kg^{-1} diet	Spleen - moderate to moderately severe lymphoid depletion	Spitsbergen et al. (1988c)
Rainbow Trout	Clophen® A50 500 mg kg^{-1} diet	Fin erosion but no lesions in spleen, head-kidney or thymus	Thuvander et al. (1991)

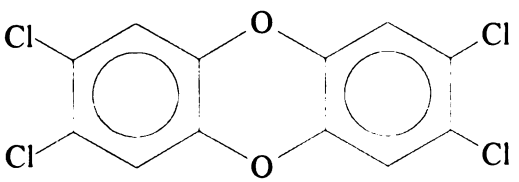
Table 2 (continued).

Species	Chemical (dose & route)	Tissue - Pathology	Reference
Rainbow Trout	Clophen® A50		Thuvander et al. (1993)
	40 mg kg^{-1} IP	No lesions in thymus or spleen	
	80 mg kg^{-1} IP	Thymus - hypocellularity (depletion of lymphoid tissue)	
		Spleen - hypocellularity	
Chinook Salmon	Aroclor ® 1254		Arkoosh et al. (1994a)
	54 mg kg^{-1} IP	No lesions in spleen or kidney	

Figure 1. Halogenated aromatic hydrocarbons (HAHs). General structure of dioxins, dibenzofurans and polychlorinated biphenyls along with representative planer congeners.

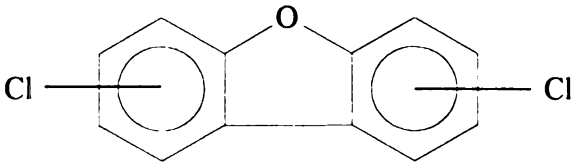
Dioxins

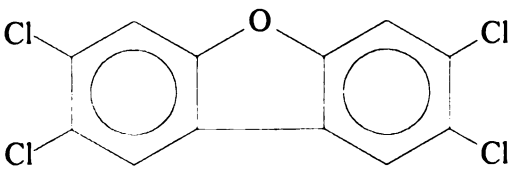




2,3,7,8-Tertachlorodibenzo-p-dioxin
(TCDD)

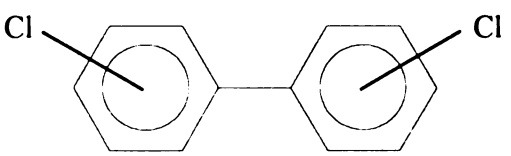
Dibenzofurans

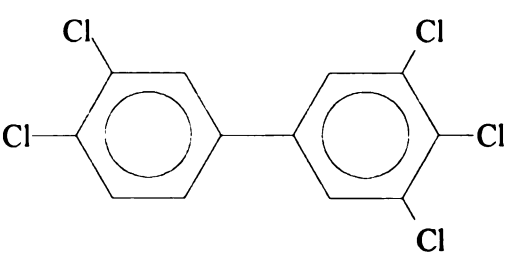




2,3,7,8-Tertachlorodibenzofuran
(TCDF)

Polychlorinated Biphenyls





3,3',4,4',5-Pentachlorobiphenyl
(PCB 126)

Figure 2. Schematic representation of the certain aspects of the teleost immune systems (Sources: Ainsworth, 1992; Evans and Jaso-Friedmann, 1992; Kaattari, 1992; Sakai, 1992; Secombes and Fletcher, 1992). Abbreviations: APC (antigen presenting cell), Ag (antigen), C (complement), IL (interlukins), MB (memory B-cell), NCC (natural cytotoxic cell), PMN (polymorphonuclear granulocytes also referred to as neutrophils).

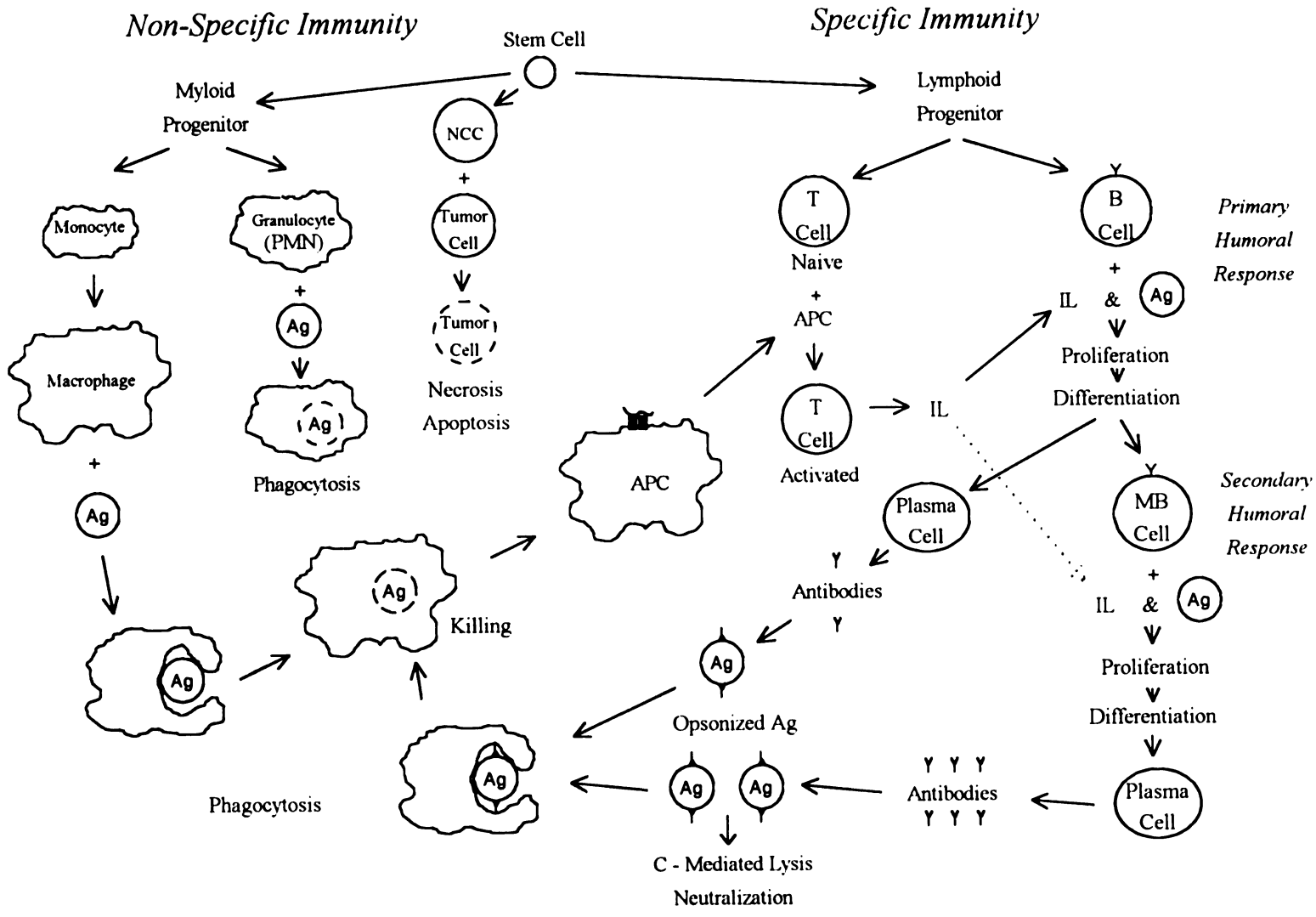
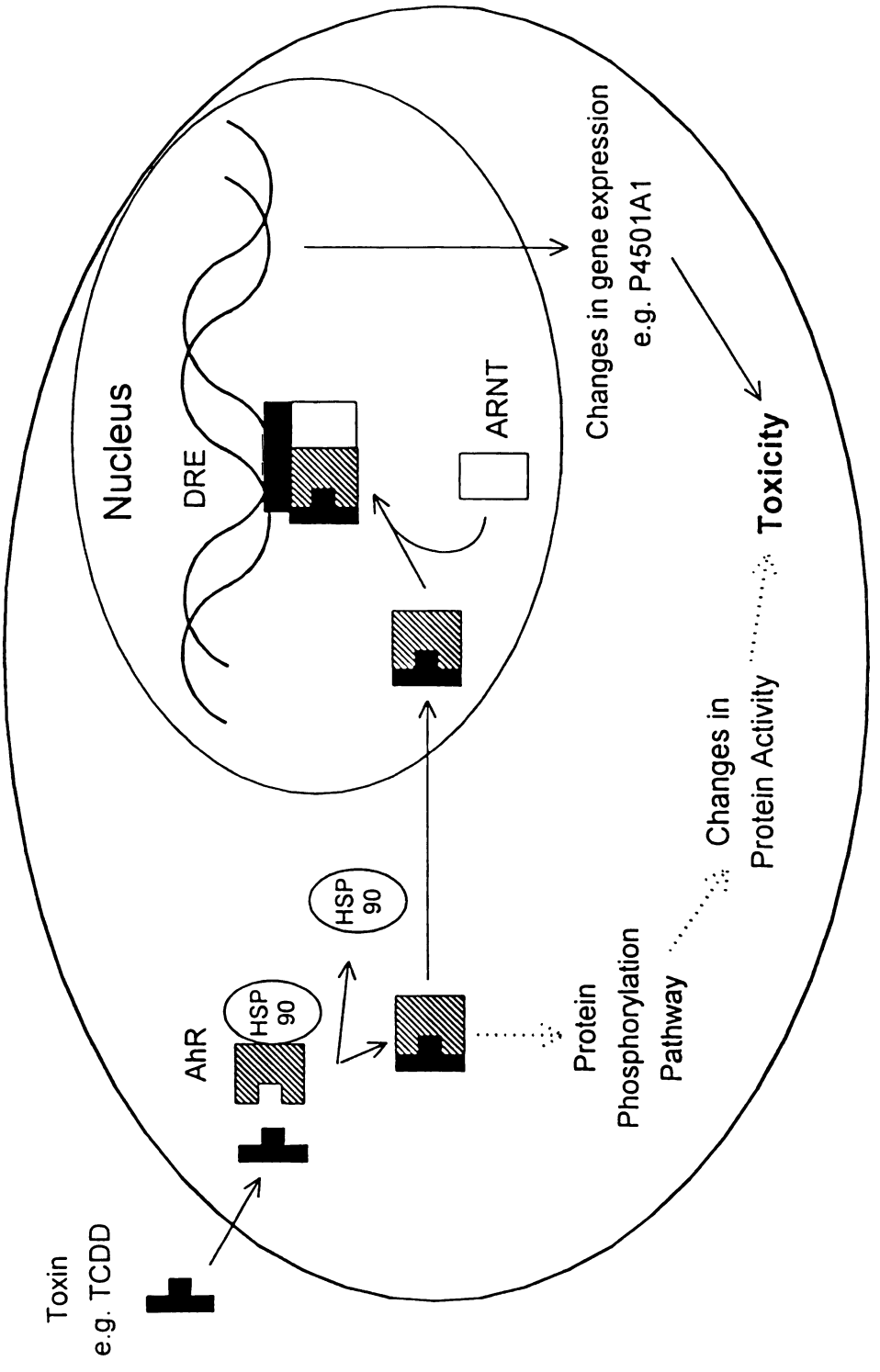

Non-Specific Immunity
Specific Immunity
Stem Cell
Myloid Progenitor
Lymphoid Progenitor
NCC
Tumor Cell
Tumor Cell
Necrosis
Apoptosis
Monocyte
Granulocyte (PMN)
Macrophage
Ag
Phagocytosis
Killing
APC
T Cell
Naive
APC
T Cell
Activated
IL
B Cell
IL & Ag
Proliferation
Differentiation
Primary Humoral Response
Plasma Cell
Antibodies
MB Cell
Secondary Humoral Response
IL & Ag
Proliferation
Differentiation
Plasma Cell
Opsonized Ag
Antibodies
C - Mediated Lysis
Neutralization
Phagocytosis

Figure 3. Proposed mechanism for Ah receptor-mediated toxins. Modified from Richter, 1995 (Sources: Whitlock, 1993; Matsumura, 1994).


Toxin
e.g. TCDD
AhR
HSP
90
HSP
90
Protein
Phosphorylation
Pathway
Changes in
Protein Activity
Toxicity
Nucleus
DRE
ARNT
Changes in gene expression
e.g. P4501A1

CHAPTER 2

In Vitro Assessment of Immunomodulatory Effects of Polychlorinated diaromatic hydrocarbons (PCDH) on Chinook Salmon, *Oncorhynchus tshawytscha*: Optimization of Lymphocyte Proliferation Assays.

(For Submission to *Fish and Shellfish Immunology*)

INTRODUCTION

Recent declines in the chinook salmon, *Oncorhynchus tshawytscha*, population in Lake Michigan have been attributed to massive die-offs of fish infected with *Renibacterium salmoninarum* (Smith, 1992), the causative agent of bacterial kidney disease (BKD). Although BKD appears to be the ultimate cause of death, other factors are thought to have played a role in rendering salmon susceptible to this otherwise ubiquitous bacteria. Organic contaminants are among the group environmental factors which have been considered as possibly contributing to the salmon mortality. Detectable levels of contaminants including, dioxins and dibenzofurans (Smith et al., 1990) and polychlorinated biphenyls (PCB) (Masnado, 1987; Smith et al., 1990; Williams et al., 1992) have been measured in chinook salmon collected from Lake Michigan waters. These polychlorinated diaromatic hydrocarbons (PCDHs) are potent mammalian immunotoxins (Vos and Luster, 1989) which

reduce host resistance to infection and disease. The potency of these compounds at suppressing immune responses in fish is less definitive because of the limited number of studies reported and apparent differences in sensitivity among species (Noguchi, 1997). An *in vitro* approach was used to assess the sensitivity of chinook salmon to the immunomodulatory effects of PCDHs. These results were used to conduct a hazard assessment of the potential contribution of these compounds to suppressed immune responses in feral salmon of the Great Lakes and potential contribution to the susceptibility of these fish to epizootics.

In vitro assays have been effective for testing the effects of drugs (Siwicki et al., 1990) and chemical contaminants (Anderson, 1992; Voccia et al., 1994) on functional immune responses in fish. Lymphocyte proliferation is one of the early events in adaptive immunity that can be measured *in vitro* by stimulating primary lymphocyte cultures with B- and T- cell mitogens. The responsiveness of lymphocytes to mitogen stimulation is a general measure of the organisms ability to mount a humoral or cell-mediated immune response. Concomitant exposure of cell cultures to mitogens and immunomodulating chemicals provides a model for assessing direct effects on lymphocyte proliferation. In order for such assays to be responsive to toxicant-related effects and not attenuated by other factors, it is critical that assay conditions which support maximal responsiveness are determined. Previous attempts to measure mitogen-induced proliferation of salmonid lymphocytes have indicated that the type of mitogen, mitogen concentration, incubation time, and source and quantity of plasma used to supplement culture media, can greatly affect the proliferative response (DeKonning and Kaattari, 1991; Leith et al., 1987; Tillitt et al., 1988).

The objective of this study was to define *in vitro* assay conditions for chinook salmon lymphocytes that support maximal mitogen-induced proliferation. Responsiveness of spleen and anterior kidney lymphocytes to mitogen stimulation was compared. The effectiveness of different media supplements (autologous and homologous salmon plasma and calf serum) and optimal mitogen concentrations and incubation times were also evaluated. In addition, factors that likely contribute to the fish-to-fish variability in mitogen responsiveness were identified.

MATERIALS AND METHODS

Fish Holding and Sampling

Juvenile chinook salmon, *Oncorhynchus tshawytscha*, were obtained from the Michigan Department of Natural Resources Wolf Lake Hatchery, Mattawan, MI and held at the National Biological Service, Great Lakes Science Center in Ann Arbor, MI. Salmon were maintained in 12° C well water and fed Biodiet, commercial salmon diet. All assays were performed using lymphoid tissues from sexually immature 1-2 year old chinook salmon. On sampling days, fish were quickly netted and sacrificed by immersion in a lethal concentation of anesthetic solution (200 mg MS-222 L^{-1}). Blood and lymphoid tissues were harvested shortly thereafter. Blood was sampled from the caudal vein by severing the caudal peduncle and was collected in heparinzed vacutainer tubes. Lymphoid tissues (spleen, anterior kidney and thymus) were removed aseptically and placed in tubes containing ice cold, RPMI-1640. Blood and tissue samples were held on ice until further processing.

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Reagents

Culture medium (RPMI-1640), antibiotics (gentamycin sulfate), mitogens (lipopolysaccharide; [LPS] and phytohemagglutinin; [PHA]) and 2-mercaptoethanol (2-Me) were purchased from Sigma, St. Louis, MO. ^{3}H-thymidine was purchased from Amersham, Arlington Heights, IL and provided by the Tissue Typing Laboratory at Michigan State University. The Warr 1.14 monoclonal antibody used for flow cytometry was obtained from the National Marine Fisheries Service, Newport, Oregon. FITC-conjugated goat anti-mouse antibody was purchased from Kirkigard and Perry, Gaithersburg, MD.

Cell Cultures

Pronephric and splenic cell suspensions were prepared by repeatedly expelling tissues from a one mL syringe (Arkoosh and Kaattari, 1992). Tubes were placed on ice for 20 min. to allow connective tissue particles to settle. The cell suspensions (supernatants) were then washed twice in RPMI-1640. After the final wash, the cells were resuspended in tissue culture medium (TCM). The basic TCM contained RPMI-1640 with L-glutamine, $NaHCO_3$ (0.2%), gentamycin sulfate (50 $\mu g\ mL^{-1}$), 2-Me (50 μM) and pH adjusted to 7.2. Various TCM supplements, including autologous or homologous salmon plasma or newborn calf serum, were assessed for their ability to sustain cell cultures and support mitogen proliferation. Salmon plasma was prepared by centrifuging heparinized blood samples (500x g at 4° C) for 10 min. Plasma from individual fish was heat-treated at 56° C for 30 min (to inactivate complement), diluted to 20% with RPMI, filter sterilized and stored at -20° C. Lymphocytes were enumerated by microscopic examination in a hemocytometer and viability was determined by trypan blue exclusion. The volume of cell suspension was adjusted to

achieve a final cell concentration of 5 x 10^6 cells mL^{-1}.

Lymphocyte Proliferation Assay

The proliferation assay was adapted from methods described by Leith et al. (1987) and DeKoning and Kaattari (1991). Ten uL of mitogen solution (LPS or PHA) was dispensed into appropriate wells of a round bottom 96 well microtiter plate (Limbro) followed by 100 ul of cell suspension. Four wells were assayed per treatment unless otherwise stated. The plates were covered with mylar plate sealers, placed in an sealed gas chamber that was purged with a 10% CO_2, 80% N_2, 10%O_2 gas mixture, and incubated at 17° C. Mitogen concentrations and incubation times were varied in order to determine conditions for maximal proliferation. ^{3}H-thymidine was added to each culture well (1.0 µCi per well) 20-24 hr before cell were harvested. The incorporation of ^{3}H-thymidine is a measure of mitotic activity. Cells were harvested using an automated cell harvester (Skatron) and the ^{3}H activity was measured by liquid scintillation and reported as counts per minute (CPM) as well as stimulation index (SI; equation 1):

$$SI = \frac{cpm\ mitogen\text{-}stimulated\ culture}{cpm\ control\ (unstimulated)\ culture} \qquad (1)$$

Flow Cytometry

Splenocyte cultures were characterized by flow cytometry by use of a modification of the method described by Thuvander et al. (1992). Cell suspensions were prepared as described previously and Ig^+ cells (B-cells) were labeled by incubating one mL of cell suspension for 45 min with Warr 1.14 monoclonal antibody (1:1000 dilution). The cells were

then wash twice and incubated for 45 min with an FITC conjugated secondary antibody (goat anti-mouse @ 5 μg mL). Cells were washed twice then resuspended in one mL RPMI-1640 a 1 x 10^6 cells mL^{-1}. Splenocyte subpopulations were distinguished based on forward and side scatter (a measure of cell surface roughness and granularity) and FITC fluorescence in a Becton Dickinson FACS-III flow cytometer. Cell suspensions were also prepared from salmon thymus in order to check the specificity of the Warr 1.14 monoclonal antibody for the Ig ligand on salmon lymphocytes and to aid in identifying the lymphocyte region of the cytograms.

Statistics

All statistical tests were performed using SYSTAT statistical software (Version 5.1), SYSTAT Inc., Evanston, IL. Effects of plasma concentration, mitogen concentration and incubation time were analyzed by one-way analysis of variance (ANOVA) on log transformed data. The effect of heterologous plasma source on lymphocyte proliferation was analyzed using the Kruskal-Wallis test. The paired sample T-test was used to analyze the effect of autologous versus homologous plasma on proliferation and Bartletts test was used to test for homogeneity.

RESULTS

Plasma Concentration

Plasma concentration significantly influenced ($P<0.001$) mitogen-induced proliferation of salmon lymphocytes (Figure 4). Maximal proliferation of splenic lymphocytes, incubated for 5 d with 100 μg LPS mL^{-1}, was observed in cultures supplemented with 1% salmon

plasma. Increasing plasma concentration from 1 to 10% resulted in a dose-related decrease in proliferation. The proliferative response was abrogated in cultures incubated with 10% plasma. Initial assays using splenocyte cultures from three salmon indicated that 1% autologous plasma supported greater proliferation (SI=48.0 ± 4.2) than culture medium supplemented with 5% newborn calf serum (SI=12.3 ± 4.0).

Mitogen Concentration

Mitogen titer was determined for both spleen and anterior kidney lymphocytes (Figure 5). Cultures were incubated for five days in TCM supplemented with 1% autologous plasma and stimulated with 12.5, 25, 50 and 100 μg LPS mL^{-1}. Mitogen responsiveness was significantly affected by LPS concentration ($P<0.001$) with lymphocytes from both tissues exhibiting maximal proliferation at 100 μg LPS mL^{-1}. Doubling the LPS concentration from 100 to 200 μg mL^{-1} did not enhance the response (data not shown). Splenic lymphocyte cultures consistently generated higher maximal responses than cultures prepared from pronephric lymphocytes.

Incubation Time

Kinetics of the proliferative response were studied by measuring ^{3}H-thymidine incorporation in splenic and pronephric cell cultures incubated for 5, 7, 9, 11 and 13 d and stimulated with 100 μg LPS mL^{-1} (Figure 6). Incubation time significantly affected ^{3}H-thymidine uptake in mitogen-stimulated cultures ($P<0.001$). The greatest stimulation occurred after 5 d, and the response decreased with continued incubation. Results from earlier tests indicated that ^{3}H-thymidine incorporation in cultures incubated for shorter periods (3 d) was

less than after 5 d.

Response to PHA

Proliferative responses to the mammalian T-cell mitogen PHA were less than the response to LPS and were also more variable. Maximum stimulation of splenic lymphocytes ranged from 2.0 - 28.9 (SI) in cultures treated for 5 d with 2.5 - 5 μg PHA mL^{-1}. Anterior kidney lymphocytes were less responsive to PHA. Maximum stimulation ranged from 0.8 to 6.26 with appreciable stimulation occurring in only in 2 of 5 fish (SI: 3.53 and 6.26 at 1.25 μg PHA mL^{-1}). Relatively great background proliferation in pronephric lymphocyte cultures (567 - 5752 cpm) contributed to the small SIs observed.

Fish-to-Fish Variability in Proliferative Response to LPS

Splenic lymphocytes from 10 individuals were cultured with optimal concentrations of autologous plasma (1%) and LPS (100 μg mL^{-1}) and incubated for the optimal time period (5 d). Even under these optimal conditions there was considerable varibility among individuals (CV 86%) with the SIs for responsive fish (8 of 10) ranging from 9.9 to 100.1 (Table 3). Two factors were identified that may contribute to the variability in mitogen responsiveness among individual fish; the source of plasma and the proportion of Ig^{-} lymphocytes (B-cells). To test for effects of plasma source, splenocytes were cultured with plasma from 12 different salmon and stimulated with LPS (100 μg mL^{-1}). The proliferative response was significantly affected ($P<0.05$) by the source of plasma with SIs ranging from 2 to 220 (Figure 7). These results indicate that soluble factors in salmon plasma can modulate responsiveness of lymphocytes and that concentrations of these factors can vary greatly

among fish. In order to reduce variability due to plasma-associated factors, plasma from several salmon were pooled and this homologous plasma was used to supplement the culture medium. The proliferative responsivness of splenocytes cultured in homologous plasma exhibited significantly less ($P<0.05$) fish-to-fish variability (CV 39%) compared to the same cells cultured in autologous plasma (CV 120%; Table 4). In addition, lymphocyte proliferation was significantly enhanced in cultures containing homologous plasma ($P<0.05$). Use of homologous rather than autologous plasma standardizes culture conditions for *in vitro* assays and reduces variability in the proliferative response of salmon lymphocytes.

Flow Cytometry

The proportion of Ig^+ lymphocytes (mature B-cells) in spleen cell suspensions from 6 individuals ranged from 31 to 62% with an average of 43% (Figure 8). Only 5.2% of thymic lymphocytes were Ig^+, which indicates little nonspecific binding of the Warr 1.14 antibody. Differences in the abundance of lymphocyte subpopulations capable of responding to a specific stimulant will likely contribute to fish-to-fish variability in lympho-proliferative responses.

DISCUSSION

The proliferative response of chinook salmon lymphocytes was sensitive to the quantity and quality of plasma used to supplement culture media. Past attempts to induce proliferation of trout and salmon lymphocytes with mitogens have employed a variety of plasma or serum supplements (Leigh et al., 1987; Sptisbergen et al., 1986; Thuvander et al., 1993; Tillitt et al., 1988; Yui and Kaattari, 1987). In studies which have used fetal calf serum

(FCS) or human serum to supplement the culture medium the proliferative response has been relatively small. The use of autologous or homologous trout plasma has been shown to be superior to FCS for supporting maximal mitogen responsiveness in rainbow trout (DeKonning and Kaattari, 1991). Similarly, we have demonstrated that salmon plasma was effective at supporting LPS-induce proliferation of chinook salmon lymphocytes. The level of stimulation was greater than in cultures supplemented with newborn calf serum and also exceeded levels previously reported for assays using FCS as a supplement (Leigh et al., 1987; Yui and Kaattari, 1987). Interestingly, the optimal plasma concentration for proliferation of chinook salmon lymphocytes (1%) was less than that for rainbow trout (10%; DeKonning and Kaattari, 1991). In fact, lymphocyte proliferation was completely inhibited in salmon splenocyte cultures containing 10% salmon plasma. These differences support recommendations by DeKonning and Kaattari (1992) that assay conditions should be optimized on a species-specific basis in order to ensure maximal responsiveness.

The chinook salmon spleen and anterior kidney harbored populations of lymphocytes that were mitogen-responsive, however, the proliferative response of splenic lymphocytes was consistently greater and less variable. Differences in mitogen responsiveness may be related to differences in cell populations. As the principal hemopoetic tissue, the anterior kidney is populated with a diversity of cells in terms of both lineage and stage of development (Zapata, 1979). Whereas, the spleen contains primarily mature lymphocytes and erythrocytes. Previous studies have demonstrated that the salmonid spleen possesses B-lymphocytes with greater affinity for antigen than the B-lymphocytes found in the anterior kidney (Irwin and Kaattari, 1986). Such lymphocyte heterogeneity indicates that organ-specific differences in lymphocyte subpopulations may be responsible for differential antigen-affinity and

responsiveness. Thus, greater proportions of mature, antigen-responsive, lymphocytes in the spleen compared to the anterior kidney, could have contributed to the greater proliferation observed in splenocyte cultures.

The responsiveness of splenic and pronephric lymphocytes to PHA was less and more variable than the response to LPS. Previous studies have also indicated that chinook salmon lymphocytes exhibit little PHA responsivenes compared to LPS and other B-cell mitogens (Leigh et al., 1987; Yui and Kaattari, 1987). This would suggest either lesser frequency and greater variability in the number of PHA-responsive lymphocytes or that assay conditions that are optimal for LPS responsiveness may not be optimal for supporting PHA-induced proliferation. Under the assay conditions described here, LPS would be superior to PHA for testing effects of immunomodulating toxins *in vitro*, because of the greater and more consistent responses.

The variability in mitogen responsiveness between individual fish was reduced by culturing cells in media supplemented with pooled homologous plasma rather than autologous salmon plasma. The use of pooled plasma helps to standardize culture conditions by eliminating the potential effect of varying concentrations of immunomodulating factors, such as cortisol or reproductive steroid hormones, that may occur in autologous plasma from different fish. Because all of the salmon used in this study were sexually immature, it is less likely that salmon plasma contained immunomodulating concentrations of reproductive hormones such as testosterone or estrogen. Although attempts were made to minimize handling stress it is possible that differences in the physiological responses of individual fish to capture resulted in varying concentrations of cortisol or other stress related hormones in the blood. Stress-induced elevation in plasma cortisol levels and associated

immunosuppressive effects is a well documented phenomena in mammals (Pruett et al., 1993) and fish (Barton and Iwama, 1991). Addition of physiologically relevant concentrations of cortisol (100 ng mL^{-1}) to lymphocyte cultures, *in vitro*, has been shown to directly suppress mitogen responsiveness (Tripp et al., 1987). Differences in plasma cortisol may have contributed to the fish-to-fish variability in mitogen responsive of lymphocytes cultured in autologous plasma (Table 4) as well as the variability in responsiveness among lymphocyte cultures supplemented with plasma from different fish (Figure 7). Further improvements in culture conditions to reduce variability and enhance responsiveness might be achieved by using carbon-stripped plasma to reduce the concentration of steroid hormones or by developing a chemically-defined plasma-free medium. Both of these approaches have been effective in mammalian cell culture.

When cell suspensions used for proliferation assays are prepared and enumerated using light microscopy it is not possible to distinguish lymphocyte subpopulations *i.e.* B-cells and T-cells. By standardizing the number of lymphocytes used in each assay (5×10^5 cells per well) it is assumed that the proportion of B- and T-cells remains constant. Thus, the number of B-cells capable of responding to LPS, for example, would be the same for each fish that is assayed. This assumption was tested by determining the proportion of Ig^+ cells (mature B-cells) in salmon spleen cell suspensions. The Warr 1.14 monoclonal antibody, previously shown to cross react with free salmon Ig, was effective for detecting sIg on salmon lymphocytes. The average proportion of Ig^+ lymphocytes in the salmon spleen (43%) was similar to values reported for rainbow trout, 28 - 35% (DeLuca et al., 1983; Thuvander et al., 1990). However, there was considerable variability among individual fish for both salmon (CV 26%) and rainbow trout (CV 26 -37%) with a two fold difference between the minimum

and maximum values in the present study. Such variability would likely contribute to differences in mitogen responsiveness among fish which could obfuscate detection of contaminant-induced changes in functional immune responses.

The use of *in vitro* assays in immunotoxicity testing should enhance the ability to detect toxicant-related effects by eliminating factors that contribute to between-fish variability in immune responses. All treatments such as multiple compounds or multiple concentrations of a single compound could be tested with cell cultures from an individual fish. Thus, treatments would be applied to cultures that are genetically identical, have the same phenotypic composition and the same physiological history. Differences in immune responses could, therefore, be more directly associated with the action of immunomodulating chemicals.

Table 3. Proliferative response of splenocytes from 10 salmon assayed under optimal mitogen concentration (100 μg LPS mL^{-1}) and incubation time (five days).

Salmon	Stimulation Index
1	37
2	14
3	1
4	100
5	1
6	63
7	71
8	63
9	35
10	10
Mean	39
SE	11
CV	86%

Table 4. Effectiveness of autologous and pooled homologous salmon plasma at supporting splenocyte proliferation. Cultures were stimulated with LPS (100 μg mL^{-1}) and incubated for five days. Results from assays using splenocytes from five individual salmon (mean ± SE).

	Stimulation Index	
	Autologous	Homologous
Salmon	Plasma	Plasma
1	1 ± 0.05	323 ± 35
2	34 ± 17	204 ± 18
3	1 ± 0.01	90 ± 11
4	114 ± 10	235 ± 5
5	182 ± 24	256 ± 9
Mean	66	222
SE	36	38
CV	120%	39%

CPM

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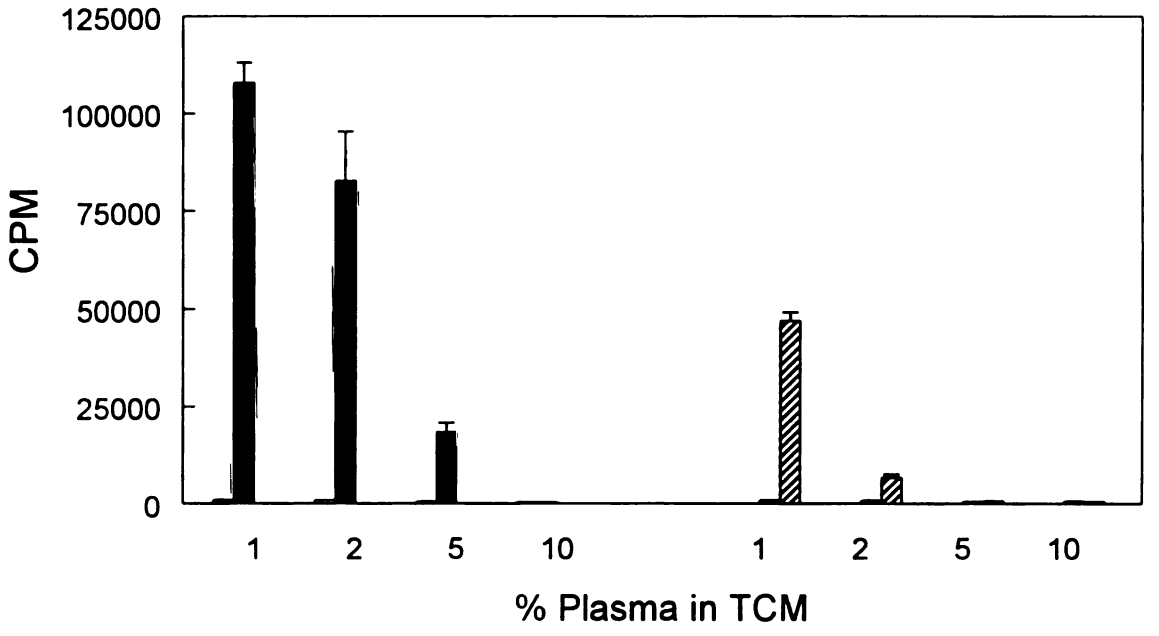


Figure 4. Influence of plasma concentration on the proliferative response (mean + SE) of splenocytes from an individual fish (filled bars) and pooled splenocytes from three fish (hatched bars) to LPS. Background proliferation in control wells is indicated by the open bars.

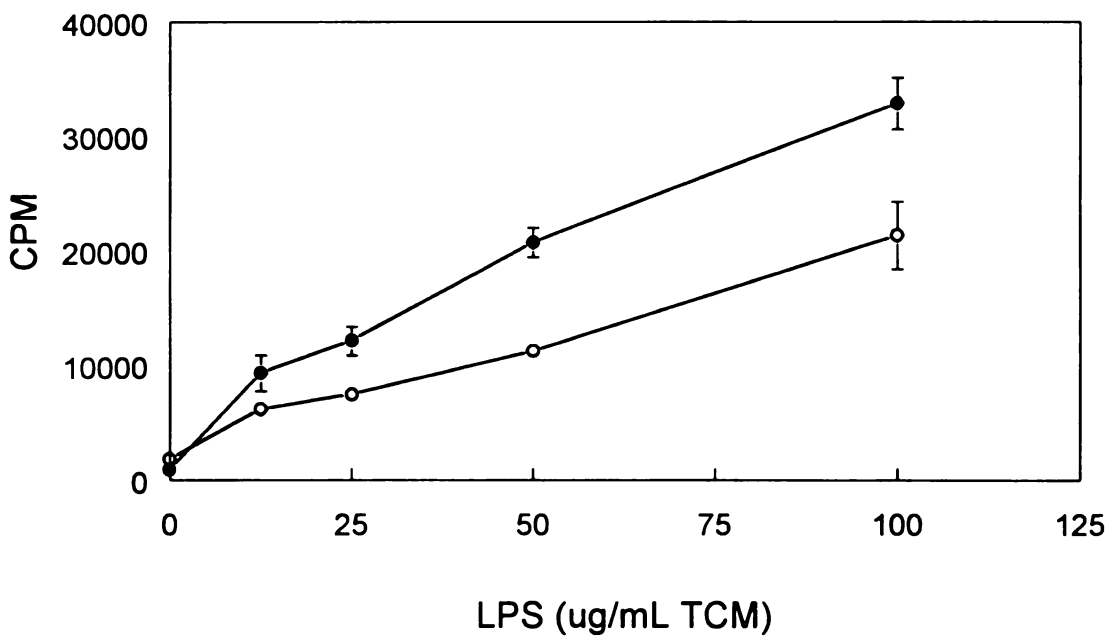


Figure 5. Effect of mitogen concentration on the proliferative response (mean ± SE) of spleen (closed circles) anterior kidney lymphocytes (open circles) to LPS.

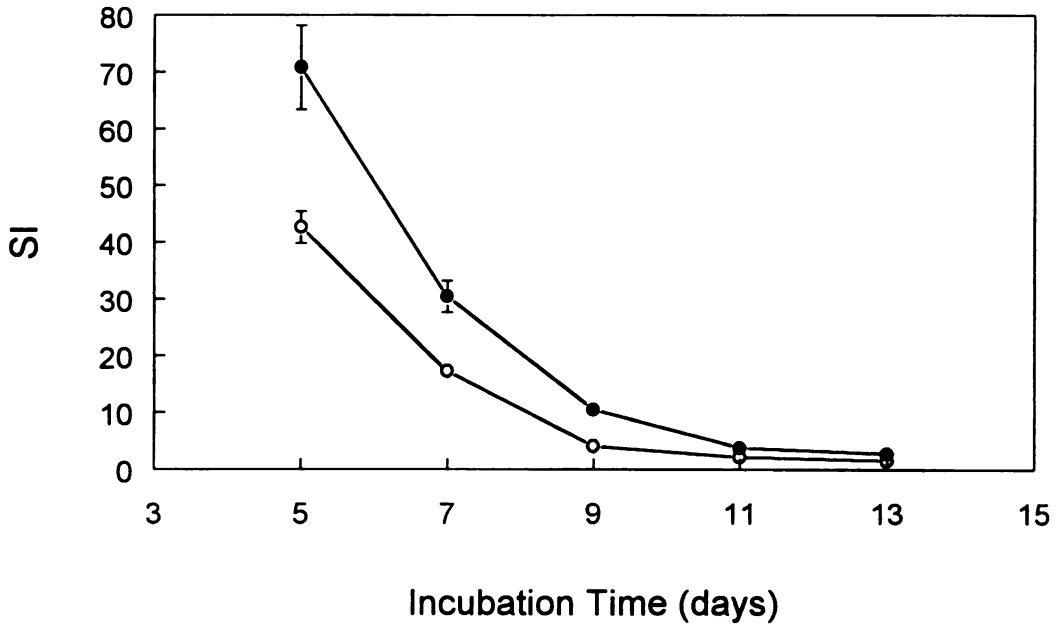


Figure 6. Kinetics of LPS-induced lymphocyte proliferation (mean ± SE) of spleen (closed circles) and anterior kidney lymphocytes (open circles).

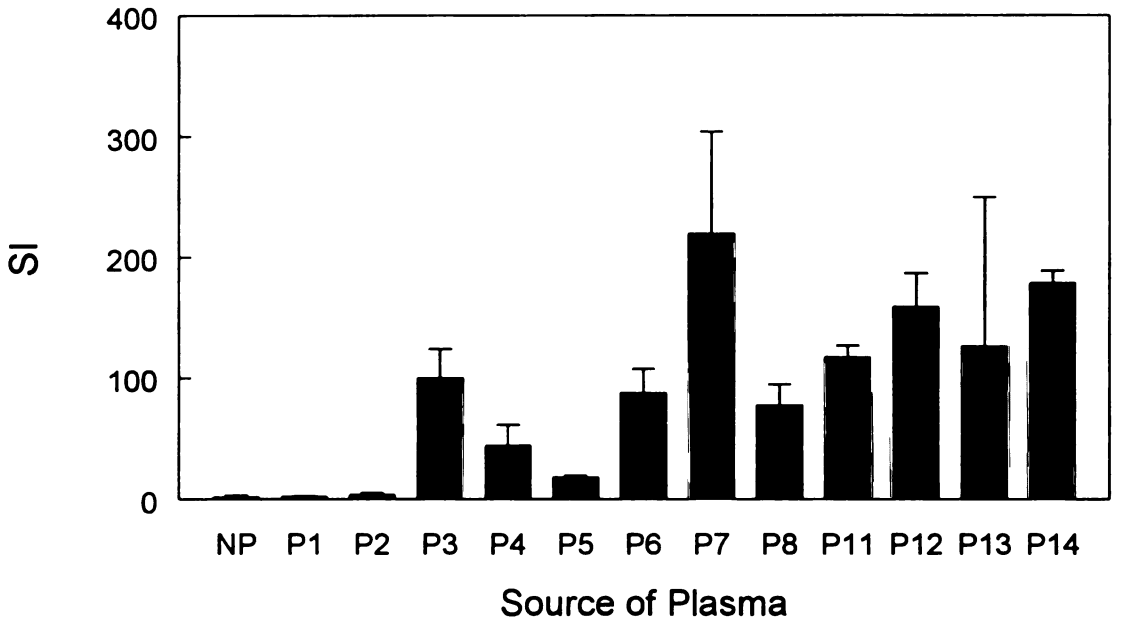
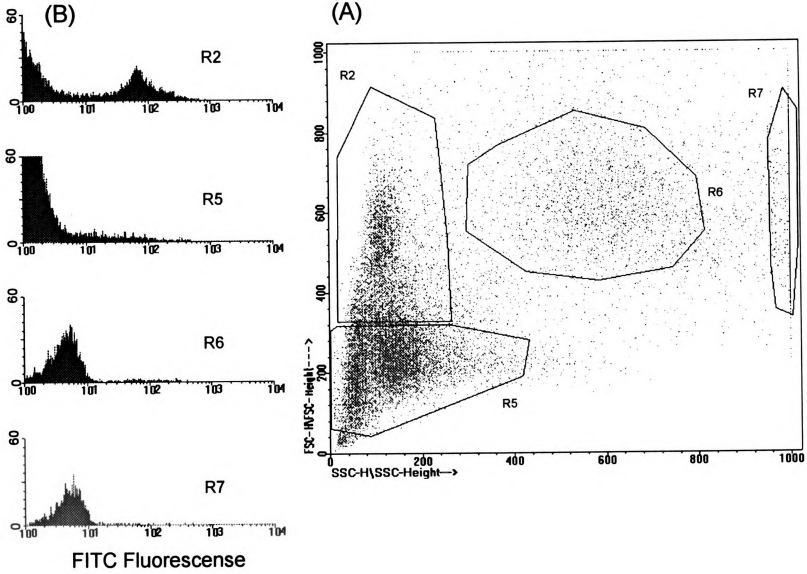


Figure 7. Effect of heterologous salmon plasma on splenocyte proliferation. Splenocytes from an individual salmon (#14) were cultured with TCM supplemented with plasma from 11 different salmon, autologous plasma (P14), no plasma (NP), and stimulated with LPS (100 ug mL^{-1}).

Figure 8. Characterization of splenocyte subpopulations using flow cytometry. A) cytogram (forward scatter vs. side scatter) of chinook salmon spleen cell suspensions. B) FITC fluorescence histograms for four gated regions from the splenocyte cytogram. Mature B-cells (Ig^+) are distinguished from Ig^- cells by FITC fluorescence, as seen in region 2 (R2).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CHAPTER 3

In vitro effects of Polychlorinated Diaromatic Hydrocarbons on Lympho-Proliferative Immune Responses in Chinook Salmon (*Onchorhynchus tschwytscha*).

(for submission to *Environmental Toxicology and Chemistry*).

INTRODUCTION

Concern over the recent epizootic outbreak of bacterial kidney disease (Smith, 1992) in Lake Michigan chinook salmon (*Oncorhynchus tshawytscha*) has raised questions about the role that chemical contaminants may play in modulating immunity and disease resistance in fish. Polychlorinated diaromatic hydrocarbons (PCDHs), including polychlorinated biphenyls (PCBs), dioxins, and dibenzofurans, are a class of anthropogenic compounds that have been detected in fish throughout the Great Lakes (Masnado, 1987; Zacharewski et al., 1989; Smith et al., 1990; Williams et al., 1992). Some PCDHs are potent mammalian immunotoxins that reduce host resistance to infection and disease (Vos and Luster, 1989). The potency of some PCDHs in suppressing immune responses in fish is less definitive. Altered immune responses have been observed in some studies (Spitsbergen et al., 1986; Thuvander et al., 1991, 1993; Rice and Schlenk, 1995) but not in others (Cleland and Sonstegard, 1987; Cleland et al., 1988a). However, recent evidence suggests that humoral

(antibody producing) immune responses in chinook salmon may be sensitive to some PCDHs. Salmon inhabiting an urban estuary in Puget Sound, contaminated with PCDHs and other anthropogenic compounds, had depressed secondary humoral immune responses (Arkoosh et al., 1991). Under controlled laboratory conditions studies showed that primary and secondary humoral immune responses were suppressed in chinook salmon treated with Aroclor 1254, a commercial polychlorinated biphenyl (PCB) mixture (Arkoosh et al., 1994a).

This study was designed to answer some fundamental questions regarding the sensitivity of chinook salmon to PCDHs by measuring direct *in vitro* effects on immune functions. *In vitro* assays have been used to identify cellular targets of PCDH immunotoxicity in mammals (Dooley and Holsapple, 1988), characterize structure activity relationships for PCDH immunotoxicity (Harper et al., 1995), and elucidate mechanisms of action (Holsapple et al., 1991). *In vitro* assays have also been effective for testing the effects of drugs (Siwicki et al., 1990) and chemical contaminants (Anderson, 1992; Voccia et al., 1994) on functional immune responses in fish. Effects measured *in vitro* may not be predictive of whole animal responses if the toxicant indirectly affects immune function by modulating other physiological systems, *e.g.* the neuroendorcrine system. However, *in vitro* assays offer several benefits including the ability to directly assess the sensitivity of target cells and immune processes. Within and among-fish variability in immune response can be estimated by studying subsamples of cells from individual fish. In this way the fish serves as its own control, thereby improving detection. *In vitro* tests also eliminate the need for containment and exposure systems that are necessary for *in vivo* studies, and require fewer fish. In addition, cellular or tissue-based effect concentrations can be predicted if toxicant concentrations in cells are measured.

Lymphocyte proliferation is one of the early events in adaptive immunity that can be measured *in vitro* by stimulating primary lymphocyte cultures with mitogens (Leith et al., 1987; Tillitt et al., 1988; DeKonning and Kaattari, 1991; Noguchi et al., 1997a). The responsiveness of lymphocytes to B-cell mitogens is a general measure of the organism's ability to mount a humoral immune response. Concomitant exposure of cell cultures to mitogens and immunomodulating chemicals provides a model for assessing direct effects on lymphocyte proliferation. The objectives of this study were to determine whether the proliferative responses of chinook salmon lymphocytes were affected by direct exposure to PCDHs (*in vitro*), to determine the immunotoxic potency of PCBs relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and to determine whether results from *in vitro* tests are consistent with, or predictive of, effects on whole fish.

MATERIALS AND METHODS

Fish Holding and Sampling

Juvenile chinook salmon were obtained from the Michigan Department of Natural Resources Wolf Lake Hatchery, MI, and held at the National Biological Service, Great Lakes Science Center in Ann Arbor, Mattawan, MI. Fish holding and sampling information have been described previously (Noguchi et al., 1997a). All assays were performed using lymphoid tissues from juvenile (220 - 420 mm) salmon.

Reagents and Chemicals

TCDD and PCB congener 126 (3,3',4,4',5-pentachlorobiphenyl) were purchased from Accurate Chemical, New Haven, CT. Aroclor 1254 was obtained from Monsanto, St Louis,

MO. Culture medium (RPMI-1640), antibiotics (gentamycin sulfate), mitogens (lipopolysaccharide-LPS), 2-mercaptoethanol (2-Me) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical, St. Louis, MO. ^{3}H-thymidine was purchased from Amersham, Arlington Heights, IL and provided by the Tissue Typing Laboratory at Michigan State University. The Warr 1.14 monoclonal antibody used for flow cytometry was obtained from the National Marine Fisheries Service, Newport, OR. FITC-conjugated goat anti-mouse antibody was purchased from Kirkigard and Perry, Gaithersburg, MD. Stock solutions of TCDD and PCBs were prepared in DMSO at concentrations 1000x greater than the nominal dosing concentration.

Cell Cultures

Splenic cell suspensions from individual salmon were prepared as described by Noguchi et al. (1997a). Briefly, the spleens were disrupted by repeated expulsion from a one mL syringe and the resulting suspension was held on ice for 20 min to allow connective tissue particles to settle. The suspended splenocytes were washed twice in RPMI-1640 and resuspended in tissue culture medium (TCM). The TCM contained RPMI-1640 with L-glutamine, $NaHCO_3$ (0.2%), gentamycin sulfate (50 μg mL^{-1}), 2-Me (50 μM), 1% pooled homologous salmon plasma and pH adjusted to 7.2. Lymphocytes were enumerated by microscopic examination in a hemocytometer and viability was determined by trypan blue exclusion. The volume of cell suspension was adjusted to achieve a final cell concentration of 5 x 10^6 cells mL^{-1}.

Lymphocyte Proliferation Assay

The proliferation assay optimized for chinook salmon lymphocytes (Noguchi et al., 1997a) was used to test for effects of PCDHs on *in vitro* mitogen responsiveness. One mL aloquots of cell suspension were dosed with 1 μL of PCDH or vehicle (DMSO). The suspension was mixed by aspirating with a repeat-dispensing pipetter. One hundred μL of cell suspension was dispensed into appropriate wells of a round-bottom 96-well microtiter plate (Limbro) containing 10 μL of mitogen solution (100 ug LPS mL^{-1}). All treatments were assayed in quadruplicate. Negative controls (cell suspension containing no LPS) and positive controls (cell suspensions containing LPS and no PCDH or vehicle) were assayed along with the treated cultures. With this dosing scheme the difference in proliferative response between replicate wells is a measure of the variability in responsiveness due to natural variability in splenocyte sensitivity and methodological factors including differences in PCDH uptake and culture conditions between wells; it does not include variability associated with dose administration, *i.e.* pipetting error. The plates were covered with mylar plate sealers, placed in a sealed gas chamber that was purged with a 10% CO_2, 80% N_2, 10%O_2 gas mixture, and incubated for four days at 17° C. ^{3}H-thymidine was added to each culture well (1.0 μCi per well) and incubated for an additional 20-24 hr before cells were harvested. Cells were harvested and the and ^{3}H activity was measured by liquid scintilation and reported as counts per minute (CPM).

Cytotoxicity

Frank cytotoxicity was determined after incubating splenocyte cultures with PCDHs for 24 hr. Cell cultures were prepared as described previously and dosed at the same

concentrations used for the proliferation assay. The number of splenocytes surviving after 24 hr was enumerated microscopically using a hemocytometer and vibility was determined by trypan blue exclusion. Cytotoxicity was determined by comparing the number of viable splenocytes in PCDH-treated cultures with untreated controls. Treatments were administered to quadruplicate cultures.

Flow Cytometry

Salmon spleocyte cultures were characterized by flow cytometry as described priviously (Noguchi et al. 1977a). Ig^+ cells (B-cells) were detected by labelling with a primary, anti-fish Ig antibody (Warr 1.14 monoclonal antibody) and a FITC conjugated secondary antibody. Splenocyte subpopulations were distinguished based on forward and side scatter and FITC fluorescence by a Becton Dickinson FACS-III flow cytometer.

Cellular Uptake of PCBs

The concentration of PCB-126 accumulated by cell cultures *in vitro* was estimated by measuring the concentration in the cell pellet. A cell suspension was prepared from salmon spleen and anterior kidney cells and adqusted to 5×10^6 cells mL^{-1}. Two-2.5 mL aliquots of cell suspension were transferred to 10 mL culture tubes dosed with PCB-126, at a nominal concentration of 100 nM. The cultures was incubated for 24 hr at 17° C then centrifuged at 500x g for 10 min. The supernatant was removed and the cell pellet was washed with fresh culture medium. The cultures were centrifuged a second time and the culture medium was removed and combined with the original supernatant. The supernatants were extracted with hexane:diethyl ether (1:1). The cell pellet was saponified with 2M alcoholic KOH and

extracted 3x with hexane:diethyl ether (1:1). Coextracted biogenic compounds, such as lipids, were removed by silica gel column chromatography. Extracts were concentrated by rotary evaporation and analyzed by GC-ECD. Cell culture dosing was performed in duplicate and culture medium without cells was dosed as a spike control. The mass of the cell pellet was estimated by multiplying the number of cells in the pellet by the cell volume and the specific gravity which was assumed to be 1.

Statistics

All statistical tests were performed using SYSTAT statistical software (Version 5.1), SYSTAT Inc., Evanston, IL. Effects of PCDHs on the proliferation of splenocytes from individual fish were analyzed by one-way analysis of variance (ANOVA) and tukeys test was used to make pairwise-comparison of treatment means. To evaluate the influence of fish-to-fish variability on the ability to detect PCDH-related effects, treatment means for assays with individual fish were combined (n=4). The combined data sets were analyzed by one-way ANOVA or by ANOVA with fish as a blocking factor, in a randomized block design. The proportion of variability explained by PCDH treatment and fish (blocking factor) was calculated by dividing the appropriate sums-of-squares (sums-of squares "PCDH" or sums-of squares "fish") by the total sums-of-squares (Neter and Wasserman, 1974).

RESULTS

Sensitivity of Splenocytes to Direct Exposure to PCDHs

Direct exposure of salmon splenocytes to PCDHs resulted in a dose-related suppression of LPS-induced proliferation. TCDD was immunosuppressive at concentrations

of 3 and 30 nM in splenocyte cultures prepared from four different salmon (Figure 9A). Because the toxicant effect was compared to unexposed (control) cultures from the same fish treatment effects were detectable despite relatively great differences in responsiveness among fish (2000 - 95000 CPM). The effect of TCDD at lesser concentrations (0.3 and 0.03 nM) was less certain. These concentrations tended to be suppressive in cultures that exhibited moderate to great LPS-responsiveness, whereas, in less responsive cultures proliferation was either not affected or was slightly enhanced. PCB-126 significantly reduced splenocyte proliferation at 1000 nM (Figure 9B). As with TCDD, the effectiveness of lesser concentrations of PCB-126 (1 - 100 nM) was more variable. Aroclor 1254 at concentraitons of 45 and 90 μM was suppressive, reducing splenocyte proliferation by >80% (Figure 9C). However, at these great concentrations Aroclor 1254 was also cytotoxic to salmon splenocytes (Figure 10). At non-cytotoxic concentrations of Aroclor 1254 (4.9 - 9 μM) suppressive effects were not consistently observed.

The cytotoxic activity of Aroclor 1254 on lymphocyte subpopulations was assessed using flow cytometry. The abundance of lymphocytes in cultures dosed with 45 uM Aroclor 1254 was less than in controls (Figure 11). In addition, the reduction in the number of Ig^+ lymphocytes (B cells) was proportionately greater than for Ig^- lymphocytes. These results suggest that lymphocyte subpopulations may be differentially affected by PCBs and that B cells may be more sensitive than Ig^- lymphocytes.

Potency of PCDHs

The relative potency of TCDD, PCB-126 and Aroclor 1254 in modulating splenocyte proliferation was estimated from dose-response curves. The effects of PCDH treatment,

expressed as a percentage of control CPM, from individual fish were averaged and plotted against log PCDH concentration (Figure 12). Typically, the toxic potency of PCDHs relative to TCDD is determined by comparing the concentrations at which the response is reduced to 50% of maximum. Because the maximum suppression by PCB-126 was less than 50%, the relative potency was calculated for concentrations at which the response was suppressed by 30% (IC_{30}). The IC_{30}, was calculated by linear interpolation from the dose-respnse curves. The IC_{30} values for TCDD, PCB-126 and Aroclor 1254 were 1.5 nM, 1.3 μM and 18.7 μM, respectively (Table 5). The potency of PCB-126 and Aroclor 1254 relative to TCDD was expressed the toxic equivalency factor (TEF). TEFs are the ratio of the TCDD IC_{30} : PCDH IC_{30} . Values >1 indicate that the compound is more toxic than TCDD and PCDHs that are less toxic have TEFs <1. The calculated TEFs indicated that PCB 126 is 1,100 times and Aroclor 1254 is 900,000 times less potent than TCDD at inhibiting salmon splenocyte proliferation.

Distinguishing Toxicant from Non-Toxicant Effects

The principal advantage to using an *in vitro* approach to assess the immunotoxicity of PCDHs was to increase the potential for detecting toxicant effects by eliminating the influence of among-fish variability. Having demonstrated direct effects of PCDHs on lymphocytes from individual fish, the next question was whether toxicant-related effects are detectable when natural variability is not restricted, in other words, what proportion of the total variability in immune response is attributable to the toxicant effect? By combining the data for replicate fish, the proportion of variability attributed to the toxicant effect and that which is due to among-fish variability was estimated. Data from four replicate fish were

combined and the effect of PCDH dose was assessed first using one-way ANOVA and subsequently by using a randomized block design ANOVA, with "Fish" as the blocking factor. When the proliferative responses (CPM) from replicate fish were analyzed together there appeared to be a dose-related depression in immune response, however, the effects were not significant for TCDD ($p=0.891$) or PCB-126 ($p=0.822$) because of relatively great among-fish variability (Figure 13A&B; Table 6). The proportion of the total variability in the proliferative response explained by these two PCDHs (sums-of-squares treatment/ total sums-of-squares) was less than 10%. Thus, greater than 90% of the variability in the proliferative response was attributed to unexplained variation ("error"; Table 6). However, when the data for the Aroclor 1254-treated cultures are analyzed together, 55% of the variability was toxicant related. This effect was statistically significant ($p=0.027$) due to the great efficacy (complete abrogation of the immune response) of Aroclor 1254 at the greatest doses tested (30 μM). When the data were blocked according to "Fish", significant toxicant effects were detected for TCDD, PCB-126 and Aroclor 1254 (Figure 13C; Table 7). Among-fish variability accounted for greater than 80% of the total variability in the responses to TCDD and PCB-126, thereby reducing the "error" to 5.4 and 4.8% respectively. Blocking was very effective at reducing "error" variance and allowed for the detection of toxicant-related effects.

Cellular Concentration of PCDHs

The *in vitro* uptake of PCDHs by salmon lymphocytes was determined by measuring the concentration of PCB-126 accumulated by pooled cultures of salmon splenic and pronephric lymphocytes. The concentration of PCB-126 in the cell pellet after 24 hr, 284 ng

PCB-126 g wet wt^{-1}, was 8.5 times greater than the dose administered to the cell suspension, 33.3 ng PCB-126 mL^{-1} (Table 8). The total recovery of PCB-126 from cells plus supernatant was 46.5%. Recovery of PCB-126 from spiked culture media (45.6%) was comparable to the recovery in cell cultures and indicates that a consistent proportion of the initial dose is not recoverable, possibly due to adsorption onto exposed surfaces of the culture tube.

In Vitro Predictions of In Vivo Effects

Results from the *in vitro* proliferation assay were compared with published reports of PCDH-related immunotoxic effects in fish dosed *in vivo*. In rainbow trout dosed with 80 mg Clophen A 50 kg^{-1} (IP), lymphocyte proliferation was reduced by 31% (Table 9). That dose is 12.9 times greater than the dose of Aroclor 1254 (6.16 μg mL^{-1}) which was found to suppress proliferation by 30% *in vitro* in this study. This might suggest that effects demonstrated *in vitro* greatly overestimate the immunotoxicity of PCBs *in vivo*. However, when the effects are related to tissue or cellular concentrations rather than dose, the assays are more similar. The estimated concentration of Aroclor 1254 accumulated by splenocytes *in vitro* (53 ug g^{-1} wet wt) was within a factor of 2 of the Clophen A 50 concentration measured in rainbow trout (30 ug g wet wt^{-1}) that were dosed with 80 mg kg wet wt^{-1} (Table 9). A similar approach was taken to compare *in vitro* and *in vivo* effects of TCDD. The dose-response relationship from a study by Spitsbergen et al. (1986) was compared with the response curve from the *in vitro* assay (Figure 14). TCDD concentrations in tissue from the *in vivo* study were estimated by multiplying the TCDD dose by 0.37; the assimilation efficiency derived for Chlophen A 50 (Table 9). Cellular concentrations from the *in vitro* assay were estimated by multiplying the TCDD dose by the 8.5 concentration factor. There

is considerable overlap between the two curves when the TCDD concentrations are expressed on a tissue or cellular basis. Although these comparisons are based on tests with different species and assume similar toxicokinetics for TCDD, PCB-126 and Aroclor 1254 and Clophen A 50 they suggest that immunotoxic effects measured *in vitro* may be useful for predicting effects in whole fish.

DISCUSSION

The use of *in vitro* assays allowed for the detection of direct effects of PCDHs on lymphocyte function. Salmon splenocytes were affected by TCDD over the same range of concentrations (3 - 30 nM) that suppress proliferative responses in TCDD-sensitive mouse strains (Karras and Holsapple, 1994). This indicates that in terms of the immunological processes involved in stimulating lymphocyte proliferation, salmon are as sensitive to TCDD as mice. However, the sensitivity to PCB-126, a non-ortho substituted (dioxin-like) PCB congener, was less in salmon (TEF=0.0011) compared to mice (TEF=0.77; calculated from data in Harper et al., 1995). Other AhR mediated effects in fish such as enzyme induction and early life stage mortality (TEFs 0.0014 - 0.027) are also less affected by PCB-126 than would be expected based on the murine model (Table 5). Thus, the structure-activity relationship (SAR) for PCDH-induced immunotoxicity in salmon appears to be consistent with other AhR-mediated effects in fish and less similar to SARs for mammals.

TCDD and PCB-126 modulated lymphocyte proliferation at concentrations that did not affect viability, whereas, immunosuppressive concentrations of Aroclor 1254 were also cytotoxic. It would appear that Aroclor 1254 affects the lympho-proliferative response through a different mechanism than TCDD or PCB-126. Because only non-ortho PCB

congeners have been effective at inducing AhR-mediated effects in fish, the dioxin-like activity of Aroclor 1254 would likely be due to the activity of non-ortho substituted congeners (#77, #81, #126 and #169). These congeners comprise 0.137% of Aroclor 1254 by weight. If all of the non-ortho congeners are considered to be as immunotoxic as PCB-126 (usually the most potent in terms of AhR-mediated effects) and their toxicity is assumed to be additive, then the sum of the non-ortho congeners in the Aroclor mixture can be compared to the potency of PCB-126 alone. This would overestimate the total potency of the non-ortho substituted PCBs. The concentration of non-ortho congeners in Aroclor 1254 at the IC_{30} would be 25.7 nM, which is about 50 times less than the IC_{30} for PCB-126 (1.3 uM). Thus, even with the over-estimate of total TCDD-like activity AhR-mediated effects induced by non-ortho congeners alone cannot account for the immunotoxicity induced by Aroclor 1254. However, estimated cellular concentrations of Aroclor 1254 that affected viability (0.38 and 0.76 mM) are in a range where nonspecific affects on cellular function, such as nonpolar narcosis, would be predicted (McCarty and Mackay, 1993). Therefore, direct effects of Aroclor 1254 on splenocyte proliferation seem to be the result of nonspecific effects on membrane function rather than effects mediated through the AhR. Results from the FACS analysis indicate that B-lymphocytes may be particularly sensitive to these nonspecific effects.

The ability to partition the variance in immune response between toxicant-related effects and "among-fish" variability, by the *in vitro* approach, enhanced the ability to statistically demonstrate PCDH-induced effects on lymphocyte proliferation. This was achieved by either analyzing dose-response relationships for individual fish separately or by combining fish but including a blocking factor ("Fish") in the analysis which increased the

homogeniety in the responsiveness of the cell populations to which the treatments were applied. This approach is particularly useful for immunological responses, such as lymphocyte proliferation, where toxicant effects may be one of several factors that modulate the response. The degree of among-fish variability frequently observed with immune responses (Spitsbergen et al., 1986; Thuvander et al., 1993: Noguchi et al., 1997a) may mask toxicant effects and increase the likelihood of Type II error, *i.e.* inability to reject the null hypothesis when in fact there were real differences.

Distinguishing toxicant-related effects with such high "background variability" is particularly challenging when treatments are applied to whole animals such as *in vivo* laboratory studies or field studies. However, there are several ways in which detection could be improved. Increasing sample size would improve the power of the test and the ability to detect toxicant effects. If the variance of the response is known or could be estimated then a simple power analysis can be used to determine the appropriate sample size required to detect a difference of a particular magnitude. For example, a sample size of 75 would be required to detect a difference of 50% given a coefficient of variation (CV) of 115%, which was the variability observed among the four fish used in the *in vitro* TCDD assays, and a 0.16 probability of a type II error. Depending on the resources available, increasing sample size in order to increasing the power of the test may not be practical. Alternatively, the immune response of individual fish could be measured before and after treatment. The effect would be measured as the difference between pre and post-treatment responses for individual fish, thus each fish would serve as its own control. However in some situations, for example field studies with feral fish or when the fish must be sacrificed in order to perform the assay, it may not be possible to measure the immune response of an individual before the treatment is

applied. In those cases, among-fish variability may be controlled using analysis of covariance (ANCOVA). This approach would require the selection of one or more covariates to measure in addition to the immunological variables. The covariates would be factors that explain some of the variability in immune response that is unrelated to the toxicant effect. These could include endogenous factors that are known to affect immune functions such as glucocorticoid or reproductive hormone concentrations (Tripp et al., 1987; Slater and Schreck, 1993). The covariate would be included in the ANCOVA model, similar to the blocking factor in the ANOVA model, to reduce the error variance and increase detection of toxicant-related effects.

Predicting immunotoxic effects on whole fish from *in vitro* assays based simply on the dose of toxicant administered leads to difficulties in interpretation because of differences between *in vivo* and *in vitro* exposure methods. In other words, it is difficult to directly compare *in vitro* doses such as nM toxicant in the medium, with *in vivo* doses such as nmol toxicant kg^{-1} fish (injected IP) or nmol toxicant kg^{-1} in the diet. A more refined approach for comparing the toxicity of different compounds using critical body residue (CBR) has been proposed by McCarty and Mackay (1992). The CBR approach uses toxicant concentrations in the organism, rather than the exposure concentration or dose, to determine potency. While this method was intended to account for pharmacokinetic differences between compounds it would also be useful for normalizing effect concentrations derived from *in vivo* and *in vitro* tests. Thus, results derived from *in vitro* and *in vivo* studies would be more comparable if the potency was based on toxicant concentrations in the cells or tissues, rather than dose or exposure concentrations.

When the CBR approach was used to normalize PCDH concentrations that affect lymphocyte proliferation, the results from the *in vitro* assays were surprisingly similar to results from *in vivo* studies performed in other laboratories. Expressing the toxic potency on a common basis enabled a more direct comparison of effect concentrations. It should be noted, however, that the calculated PCDH concentrations in cells (*in vitro*) and tissues (*in vivo*) were based on several assumptions and estimations. For example, the *in vitro* concentration factor (8.5) for PCB-126 was determined using a culture system (culture tubes) that was greater in scale that the 96-well microplate format used for the *in vitro* assays. Differences in the surface area to volume ratios of the two systems may have slightly affected the proportion of the PCB dose that was available for cellular uptake. Also, the mass of cells in the culture tube was estimated from the number of cells, their calculated volume and their specific gravity which was assumed to be about 1. In addition, cellular concentrations of TCDD and Aroclor 1254 were calculated using the *in vitro* concentration factor derived for PCB-126. The partitioning of PCDHs between culture media and cells, *in vitro*, would be influenced primarily by the octanol:water partition coefficient (K_{ow}) of the PCDH. Because the log K_{ow} for PCB-126 (6.89; Hawker and Connell, 1988) is similar to TCDD (6.15 - 7.28; Jackson et al., 1986) and Aroclor 1254 (6.1 - 6.8; Mackay et al., 1983), the 8.5 value would be a reasonable estimation of the concentration factors for TCDD and Aroclor 1254. It was also assumed that the concentration factor, determined at 100 nM, would remain constant over the range of PCDH concentrations tested (0.3 nM - 90 uM), i.e. the uptake curve is linear. Departures from linearity may occur at the greater exposure concentrations if uptake follows the Michaelis-Menton model of saturation kinetics. If this occurs then applying a constant concentration factor may tend to overestimate cellular concentrations at greater

exposure concentrations. In terms of the tissue concentrations, the assimilation efficiency derived from the Thuvander et al. (1993) study for Aroclor 1254 (0.37) was used to estimate TCDD tissue concentrations from the Spitsbergen et al. (1986) study based on the dose of TCDD administered. Considering the relatively short exposure duration (6 weeks) and similar half lives of TCDD (105 days; Kleeman et al., 1986) and PCBs (219 days; Nimii and Oliver, 1983) it would appear that the 0.37 assimilation efficiency would provide an adequate approximation of the expected TCDD tissue concentrations. Overall, the assumptions and estimations required to calculate cellular and tissue concentrations would not appear to be extraordinary.

Having determined the potency with which PCDHs modulate lymphocyte proliferation it was possible to predict whether current concentrations in feral chinook salmon are likely to be affecting immune responses. The likelihood that immunotoxic effects may occur was based on the quotient method; the ratio of the PCDH concentration in the fish divided by effect concentration derived from the *in vitro* tests (Table 10). For this calculation, the IC_{30} for each PCDH was multiplied by the concentration factor (8.5) to estimate the cellular concentration at the IC_{30}. Ratios greater than 1 indicate that the concentrations in the fish exceed the effect concentration and there is a risk that the effect will be realized, whereas, values less than 1 indicate little risk of occurrence. The likelihood that TCDD or PCB-126 alone are affecting lympho-proliferative responses would be negligible because their ratios were <0.0001 (Table 10). The ratios for Aroclor 1254 (0.019 - 0.076) were about 100 times greater than for TCDD and PCB-126, but well below 1. The greatest dose of Aroclor 1254 that did not consistently affect the proliferative response was 9 μM; estimated to be 25 μg/g in the cells. This concentration is 6 fold greater than Aroclor 1254 concentration in salmon

tissues and suggests that PCB concentrations are below the threshold for effects on lymphocyte proliferation.

CONCLUSIONS

The lymphocyte proliferation assay provided a means of measuring direct effects of PCDHs on salmon immune functions, *in vitro*, and improved the ability to distinguish toxicant from non-toxicant effects. The toxic potency of PCDHs derived from the *in vitro* assays compared favorably with potencies determined from *in vivo* studies. Concentrations of TCDD, PCB-126 and Aroclor 1254 in feral chinook salmon from Lake Michigan are below the predicted effect concentrations derived from *in vitro* assays and would present a low risk in terms of direct effects on lymphocyte proliferation.

Table 5. Toxic equivalency factors (TEFs) calculated for the salmon splenocyte proliferation assay and other Ah-R mediated effects in fish. TEF = PCB IC_{30} / TCDD IC_{30}.

PCDH	Lymphocyte IC 30	proliferation TEF	Juvenile rainbow trout EROD TEF[1]	Rainbow trout AHH TEF[2]	Lake trout early life stage mortality TEF[3]	RLT bioassay TEF[4]
TCDD	1.5 nM	1	1	1	1	1
PCB 126	1.3 uM	0.0011	0.0014	0.005	0.005	0.027
Aroclor 1254	18.7 uM	0.00009				

[1] from Newsted et al., 1995
[2] from Janz and Metcalfe, 1991
[3] from Walker and Peterson, 1991
[4] from Richter et al., 1996

Table 6. Analysis of variance table for one-way ANOVA tests for effects of PCDH treatment on lymphocyte proliferation when replicate fish are analyzed together (n = 4 for each compound). The percent of total variability explained by different sources is the ratio of source sums-of-squares : total sums-of-squares.

Source	Sums of Squares	df	Mean Square	F-Ratio	P	Percent of Total Variability
TCDD	0.0998E10	4	2.49E8	0.272	0.891	6.8
Error	1.37E10	15	9.16E8			93
Total	1.47E10					
PCB 126	0.345E10	4	0.862E9	0.377	0.822	9.1
Error	3.44E10	15	2.29E9			91
Total	3.78E10					
A1254	3.25E10	2	1.62E10	5.538	0.027	55
Error	2.64E10	9	0.293E10			45
Total	5.88E10					

Table 7. Analysis of variance table for randomized block ANOVA to test for effects of PCDH treatment on lymphocyte proliferation when replicate fish are analyzed together (n = 4 for each compound). Fish number was used as the blocking factor. The percent of total variability explained by different sources is the ratio of source sums-of-squares : total sums-of-squares.

Source	Sums of Squares	df	Mean Square	F-Ratio	P	Percent of Total Variability
FISH	1.29E10	3	4.31E9	65.1	<0.001	0.88
TCDD	0.0998E10	4	0.249E9	3.77	0.033	0.068
Error	0.0794E10	12	0.0662E9			0.054
Total	1.47E10					
FISH	3.25E10	3	1.08E10	71.2	<0.001	0.86
PCB 126	0.345E10	4	0.0862E10	5.7	0.008	0.091
Error	0.183E10	12	0.0152E10			0.049
Total	3.78E10					
FISH	1.71E10	3	0.569E10	3.66	0.083	0.290
A1254	3.25E10	2	1.62E10	10.4	0.011	0.552
Error	0.932E10	6	0.155E10			0.158
Total	5.88E10					

Table 8. Distribution of PCB 126 in cell cultures 24 hours after dosing with 33 ng PCB 126/mL. Results are from duplicate cultures, mean (SE).

Matrix	ng PCB 126 in matrix	Recovery (%)	Concentration in Matrix	Ratio: Conc. : Dose
Supernatant	27.8 (3.7)	33.4	11.1 ng/mL	0.33
Cells	11.1 (1.3)	13.2	284 ng/g	8.5
Supernatant + Cells	38.8 (4.9)	46.5		
Spiked TCM	38.0 (8.5)	45.6		
Blank	not detected			

Table 9. Comparison of *in vitro* and *in vivo* approaches to evaluating the effect of PCBs on lymphocyte proliferation. Cellular concentrations of Aroclor 1254 were estimated from the *in vitro* assay at the IC_{30}, and compared to the concentration of Clophen A50 measured in rainbow trout that were dosed (IP) with 80 mg/kg (Thuvander et al., 1993). Proliferation of rainbow trout splenocytes to LPS in Clophen A50-treated fish was depressed by 31% compared to controls, although the effect was not statistically significant in that study. The assimilation efficiency for Clophen A50 was calculated, based on data from the Thuvander study, as the ratio of Clophen A50 concentration measured tissues : dose of Clophen A50 administered.

	In vitro assay	*In vivo* test (Thuvander et al., 1993)
Species:	chinook salmon	rainbow trout
Dose Administered:	6.2 µg Aroclor 1254/mL	80 mg Clophen A50/mg (IP)
Assimilation Efficiency:		0.37
Concentration Factor:	8.5	
Concentration in cells or tissues:	53 µg/g	30 µg/g
Effect on Proliferation:	-30%	-31%

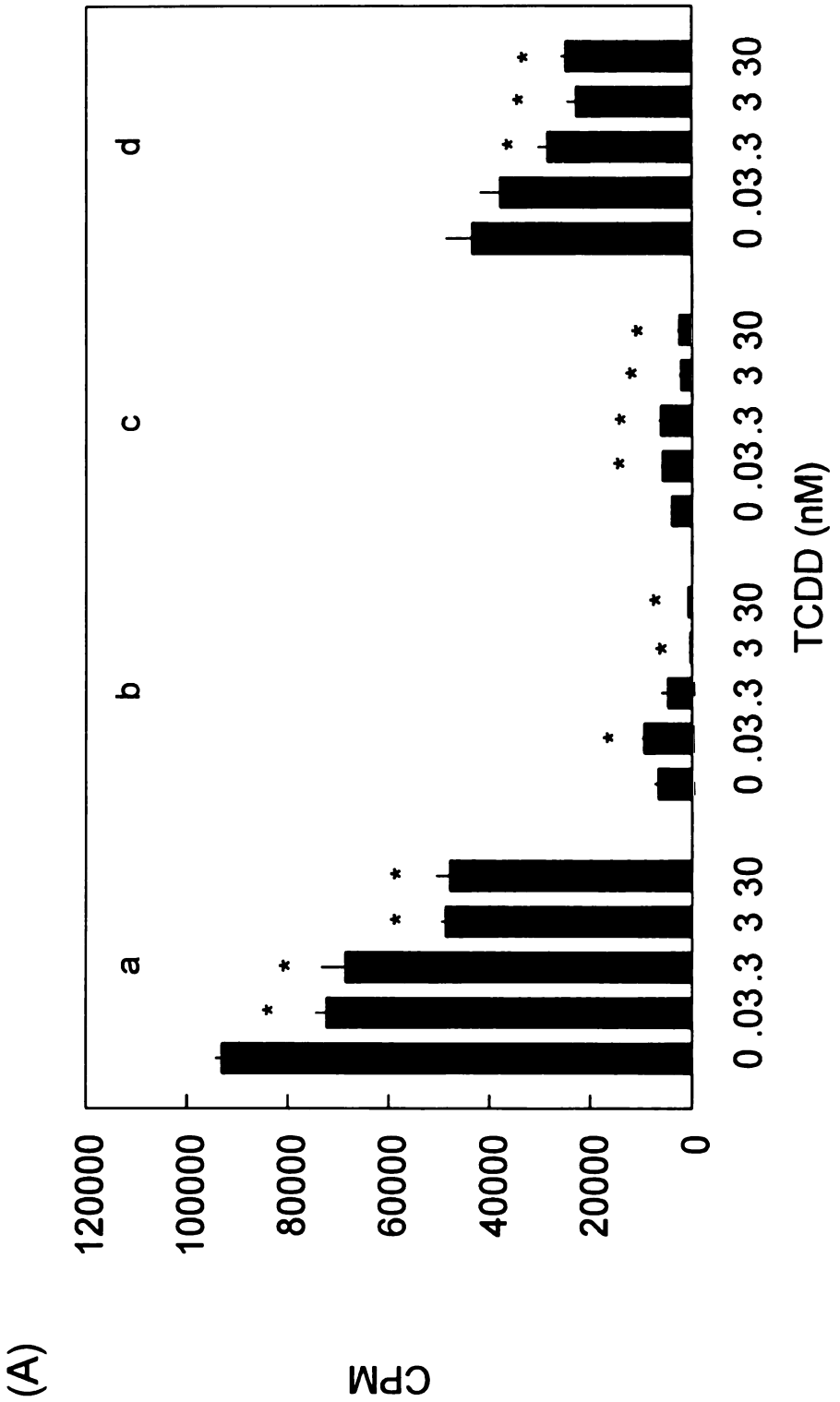
Table 10. Comparison of environmental concentrations of PCDHs in Lake Michigan chinook salmon with estimated effect concentrations based on the *in vitro* splenocyte proliferation assay.

PCDH	Concentration in Lake Michigan Chinook Salmon	Estimated cellular concentraiton at the IC_{30}	Ratio: Environmental conc./ Effect Conc
Aroclor 1254	1 - 4 μg/g[1]	53 μg/g	0.019 - 0.076
PCB 126	0.5 - 1.3 ng/g[1]	3048 ng/g	0.0002 - 0.0004
TCDD	3 pg/g[2]	4624 pg/g	0.0006

[1] G. Noguchi (unpublished data)

[2] Smith et al., 1990

Figure 9. Proliferation of salmon splenocyte cultures stimulated with LPS and treated with graded doses of TCDD (A), PCB-126 (B) and Aroclor 1254 (C). Results are from *in vitro* assays with cells from four individual fish (denoted by lower case letters a, b, c, etc.). Values are means±SE for 4 replicate cultures per treatment. Significant differences (α=0.05) between PCDH-treated cultures and controls are indicated by an asterisk (*). Proliferation in control cultures (vehicle alone) was not significantly different from positive control cultures (no vehicle). Background proliferation (cpm) in non-stimulated cultures (no LPS) from individual fish were 562 (a), 252 (b), 238 (c), 683 (d), 769 (e), 906 (f), 847 (g), 1438 (h), 4670 (i), 1232 (j), 550 (k), and 951 (l).


(A)
CPM
120000
100000
80000
60000
40000
20000
0
a
b
c
d
0 .03 .3 3 30
TCDD (nM)

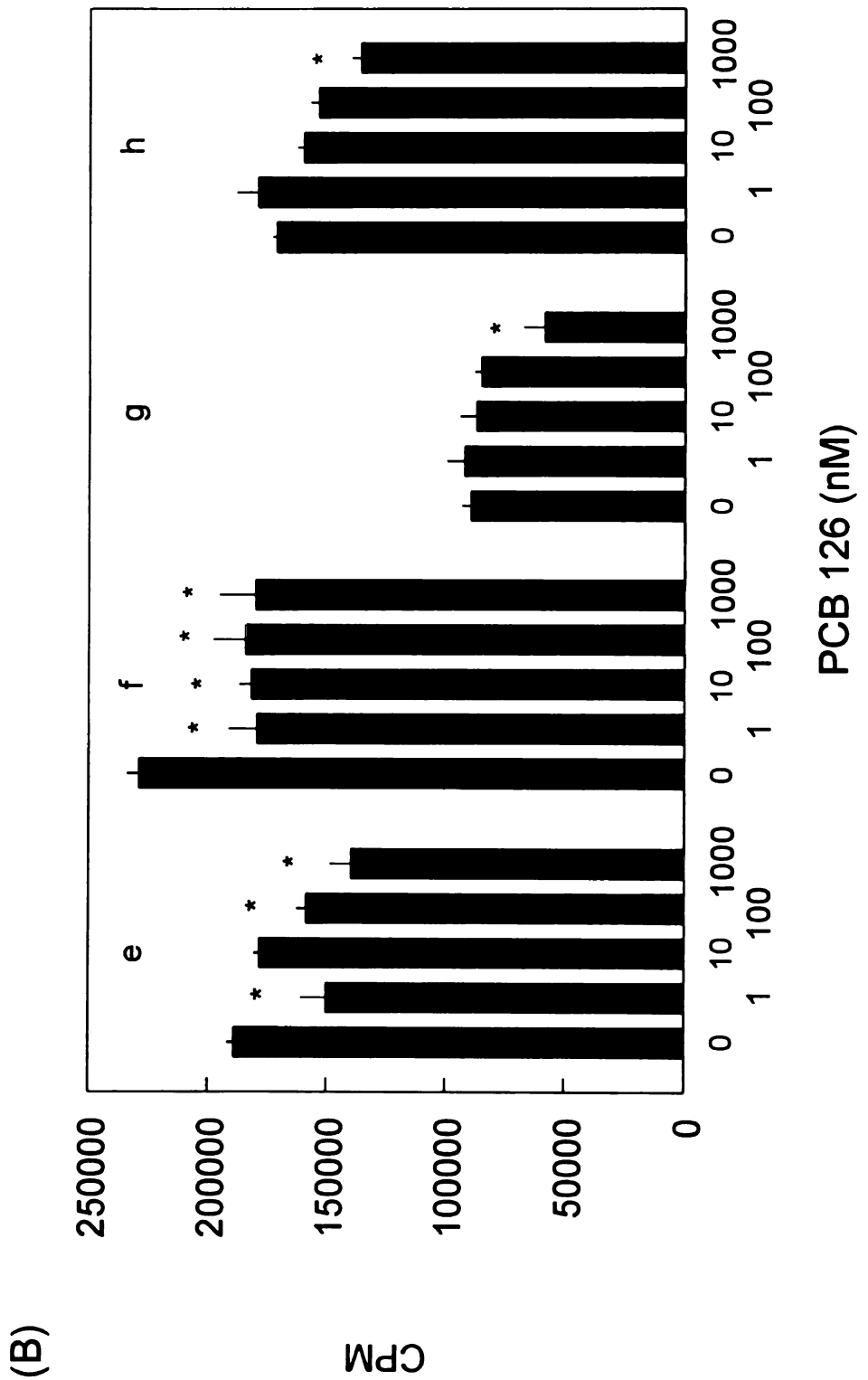


Figure 9 (continued).

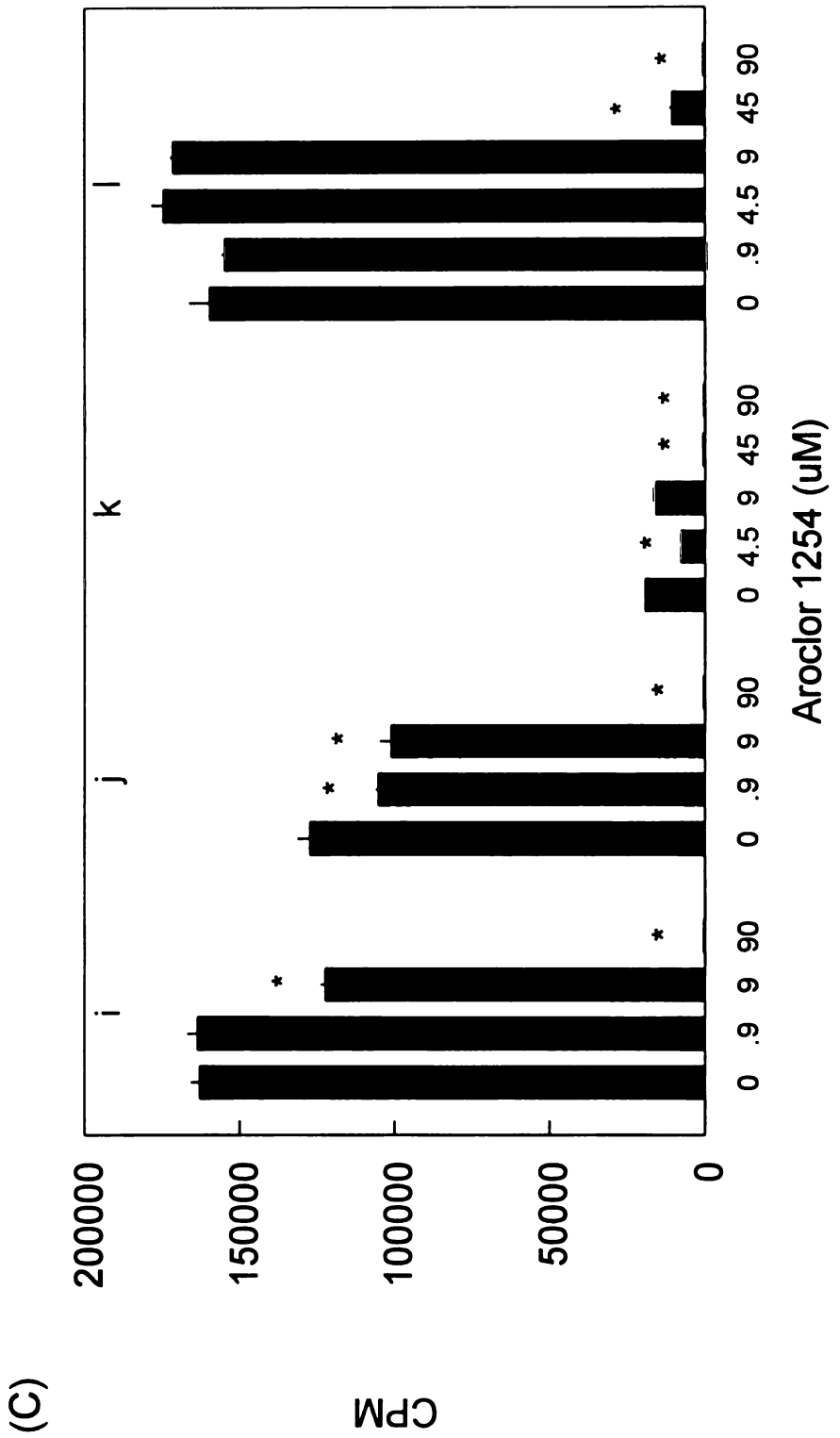


Figure 9 (continued).

Figure 10. Cytotoxicity of TCDD, PCB-126 and Aroclor 1254 to chinook salmon splenocytes. Numbers of viable splenocytes were counted after 24 hr incubation. Values are means±SE for 4 replicate cultures per treatment. Significant differences (α=0.05) between PCDH-treated cultures and controls are indicated by an asterisk (*).

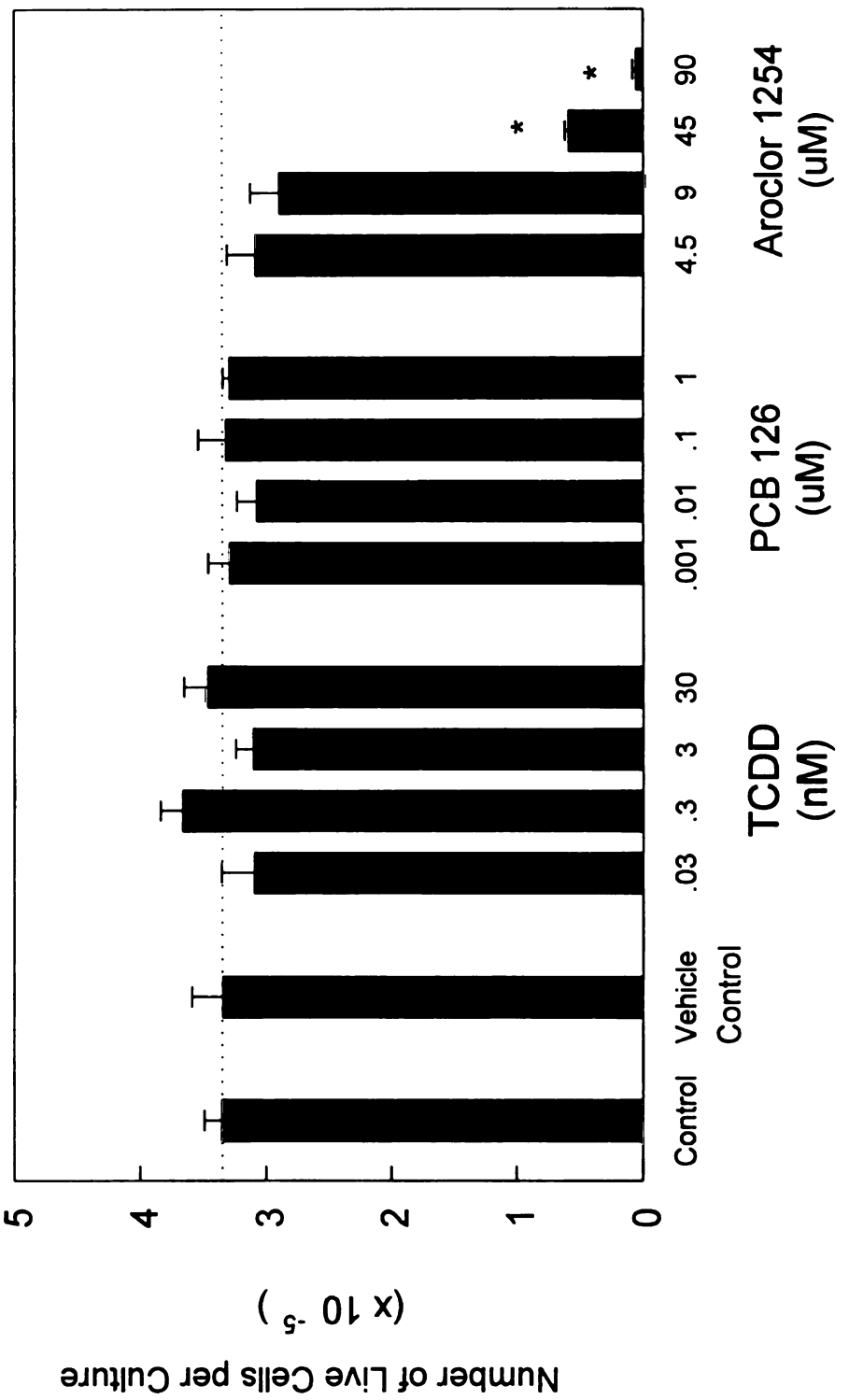

Number of Live Cells per Culture
(x 10 -5)
0
1
2
3
4
5
Control
Vehicle Control
.03
.3
3
30
.001
.01
.1
1
4.5
9
45
90
TCDD (nM)
PCB 126 (uM)
Aroclor 1254 (uM)
*
*

Figure 11. Characterization of the effects of Aroclor 1254 on splenocyte subpopulations using flow cytometry. A) cytograms (forward scatter vs. side scatter) and B) FITC fluorescence in the lymphocyte region (R2) of cell suspensions dosed with 0, 9 and 45 μM Aroclor 1254. C) Percentage of Ig^+ lymphocytes (B cells) in the lymphocyte region, based on FITC fluorescense.

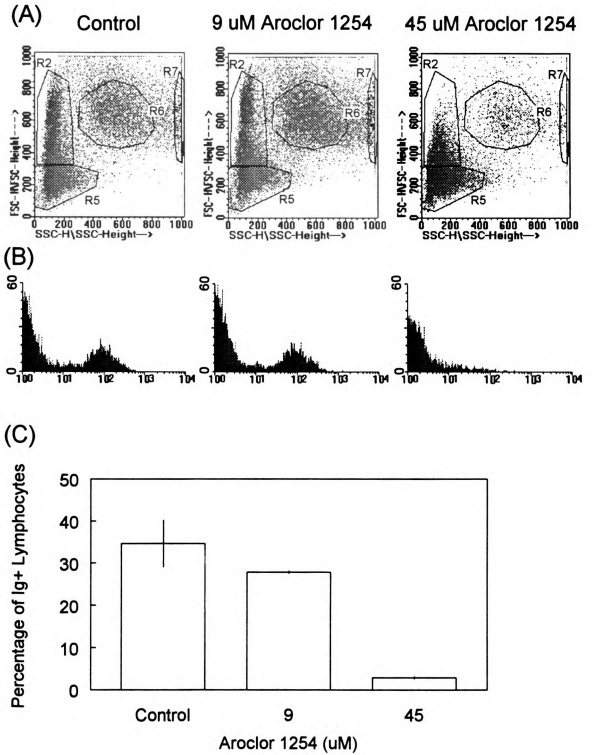
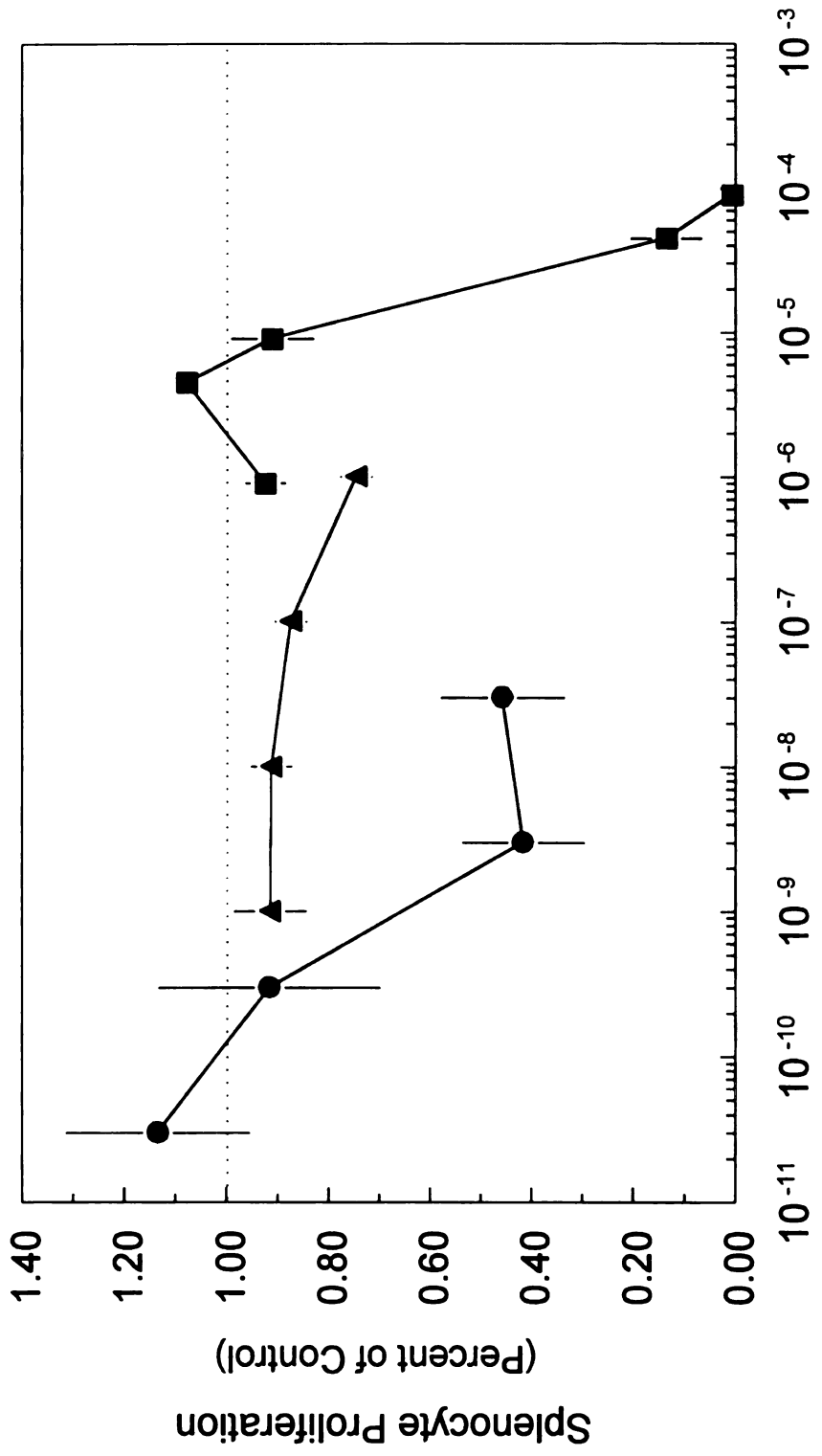

(A)
Control
9 uM Aroclor 1254
45 uM Aroclor 1254
R2
R7
R6
R5
FSC-H\FSC-Height-->
SSC-H\SSC-Height-->
(B)
(C)
Percentage of Ig+ Lymphocytes
Control
9
45
Aroclor 1254 (uM)

Figure 12. Dose-response curves for the effects of TCDD (●), PCB 126 (▲) and Aroclor 1254 (■) on chinook salmon splenocyte proliferation. Responses, expressed as percent of control proliferation (cpm), from 4 replicate fish were combined and plotted (mean±SE) against log PCDH concentration (M).


Splenocyte Proliferation
(Percent of Control)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4
10-3
PCDH Concentration (M)

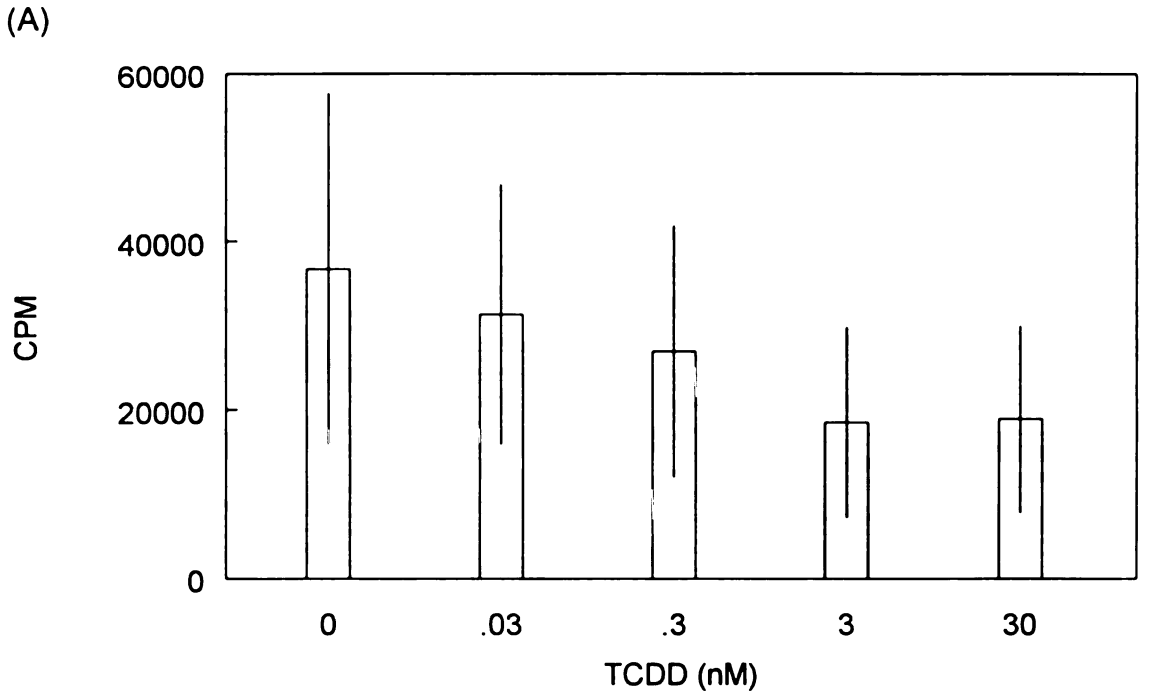
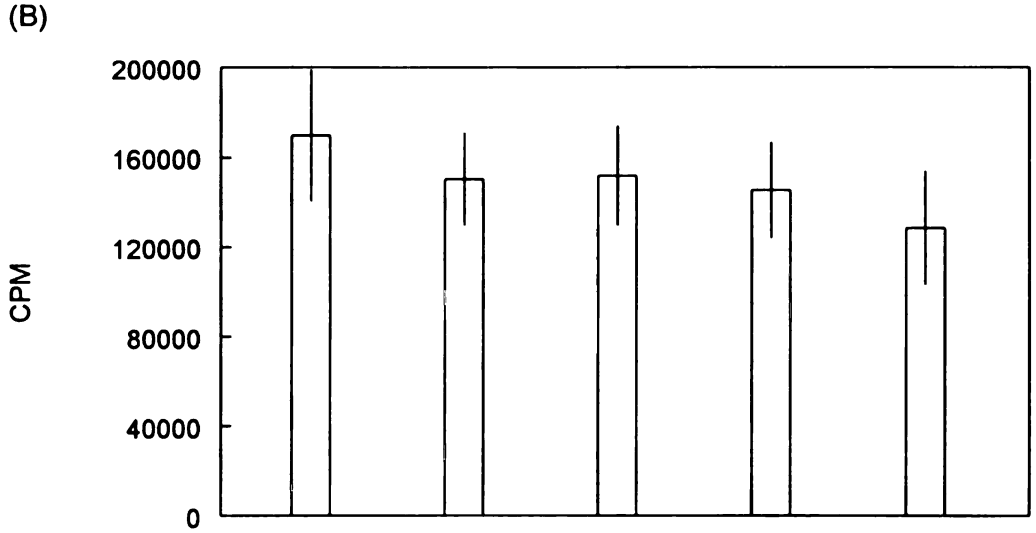


Figure 13. Direct effects of TCDD (A), PCB 126 (B) and Aroclor 1254 (C) on splenocyte proliferation with replicate fish combined. Values are means±SE for the 4 replicate fish shown in Figure 9.

(B)



(C)

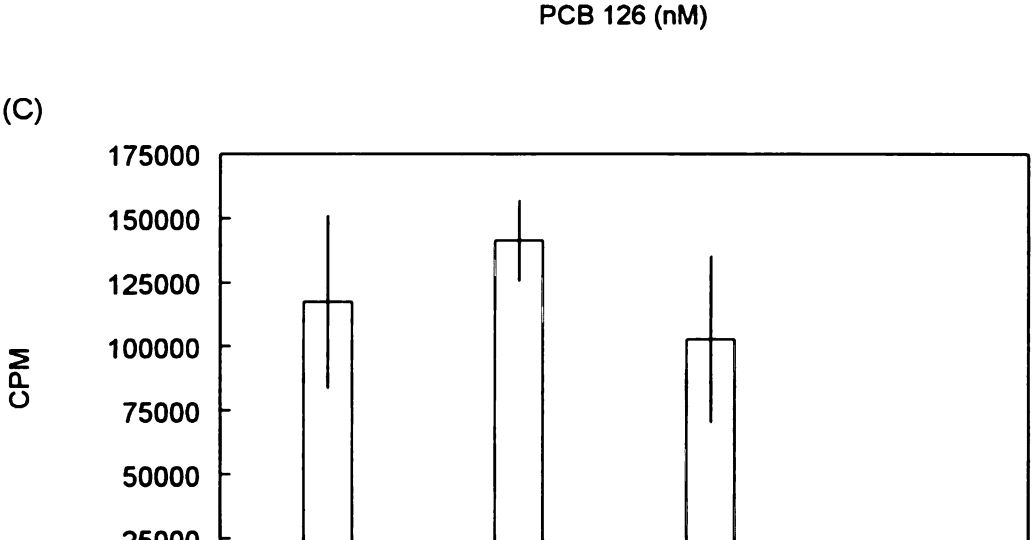


Figure 13 (continued).

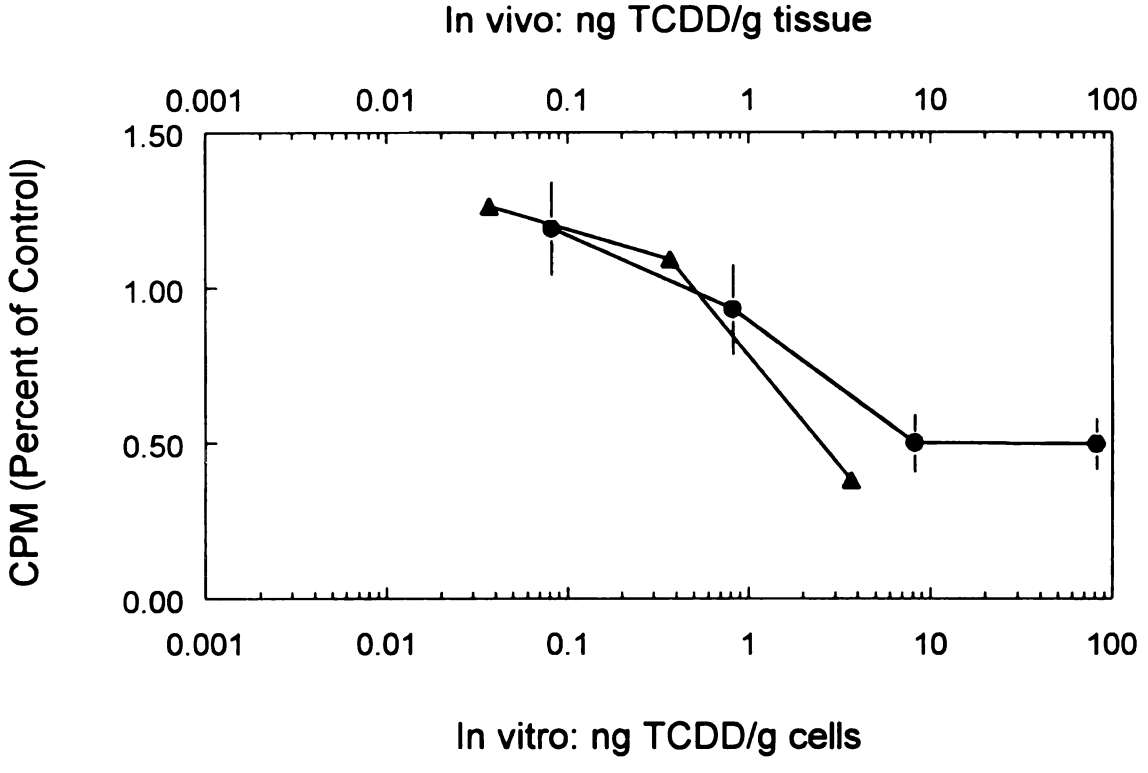


Figure 14. Comparison of *in vivo* and *in vitro* effects of TCDD on lymphocyte proliferation (CPM). The dose-response curve for *in vitro* effects (●) is based on *in vitro* assays with chinook salmon splenocytes. Data from Spitsbergen et al., (1986) were used to construct the dose-response curve for *in vivo* effects of TCDD on splenocyte proliferation in rainbow trout (▲).

CHAPTER 4

Development of an Assay to Assess Effects of Polychlorinated Diaromatic Hydrocarbons on *In Vitro* Antibody Production and Antibody Affinity in Chinook Salmon (*Onchorhynchus tschwytscha*) Splenocyte Cultures.

(for submission to *Developmental and Comparative Immunology*)

INTRODUCTION

Polychlorinated diaromatic hydrocarbons (PCDH) comprise a class of chemicals such as, polychlorinated biphenyls (PCB), polychlorinated dibenzo-p-dioxins (dioxins) and polychlorinated dibenzofurans (PCDF), that induce pleiotropic effects in mammals including immunomodulation (Vos and Luster, 1989). The humoral or antibody producing immune response in adult and juvenile rodents is particularly sensitive to some of these compounds (Holsapple et al., 1991b) and PCDHs can also affect humoral immunity in fish (Thuvander et al., 1991; Arkoosh et al., 1994a; Rice and Schlenk, 1995). The method most frequently used to measure humoral immune responses in fish and mammals is the antibody-forming-cell (AFC) assay, also referred to as the plaque-forming-cell (PFC) assay. The AFC assay detects the number of B cells that have been stimulated to produce antibodies in response to contact with antigen. Another method for detecting humoral responses is to measure the quantity of

antibody produced. The advent of enzyme-linked immunosorbant assay (ELISA) technology has lead to the development of sensitive and reproducible quantitative assays for the detection of serum antibodies (Hornbeck et al., 1991). Determination of antibody production by ELISA has been shown to be as sensitive as the AFC response for detecting effects of immunomodulating chemicals on humoral immunity in rodents (Temple et al., 1993). ELISA protocols have been adapted for the detection of antibodies produced by fish (Arkoosh and Kaattari, 1990).

ELISAs have also been developed to characterize the binding affinity of antibodies for antigen (Nieto et al., 1984). An affinity ELISA has recently been adapted for assessing the affinity of antibodies produced by fish (Shapiro, 1995). Results from that study have demonstrated that affinity maturation, a critical element of the memory responses in mammals, also occurs in fish. Thus, analysis of serum antibodies by ELISA can be used to detect not only the magnitude of the humoral response, but to also characterize the nature of the response, *i.e.* primary versus secondary responses.

Elements of the humoral immune response including the induction of antibody production have been reproduced *in vitro* using primary cultures of human (Wood et al., 1993), rodent (Morris et al., 1993) and fish (Kaattari and Yui, 1987; Shapiro, 1995) lymphocytes. An *in vitro* assay system for antibody production coupled with quantitative and affinity ELISA for antibody detection could provide a sensitive and informative system for assessing effects of PCDHs on humoral immunity in fish. The objectives this study were to evaluate the effects PCBs and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on humoral immunity in chinook salmon. Lymphocyte culture techniques were adapted to support antibody production by splenocyte cultures, *in vitro.* Antibodies produced in culture

supernatants were detected by quantitative ELISA and the affinity of antigen-specific antibodies was determined by affinity ELISA.

MATERIALS AND METHODS

Fish Holding and Sampling

Juvenile chinook salmon were obtained from the Michigan Department of Natural Resources Wolf Lake Hatchery, Mattawan, MI, and held at the National Biological Service, Great Lakes Science Center in Ann Arbor, MI. Fish holding and sampling information were described previously (Noguchi et al., 1997a). All assays were performed using splenic cell suspensions from juvenile (200 - 300 mm) chinook salmon salmon.

Reagents and Chemicals

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and 3,3',4,4',5-pentachlorobiphenyl (IUPAC #: PCB-126) were purchased from Accurate Chemical CO, New Haven, CT. Aroclor 1254 was obtained from Monsanto, St Louis, MO. Culture medium (RPMI-1640), antibiotics (gentamycin sulfate), trinitrophenyl-haptenated lipopolysaccharide (TNP-LPS), 2-mercaptoethanol, dimethylsulfoxide (DMSO), were purchased from Sigma Chemical, St. Louis, MO. The Warr 1.14 monoclonal antibody used in the quantitative and affinity ELISA was obtained from the Virginia Institute of Marine Sciences, College of William and Mary, Gloucester Point, VA and the HRP-conjugated goat anti-mouse secondary antibody was purchased from Kirkigard and Perry, Gaithersburg, MD. Stock solutions of TCDD and PCBs were prepared in DMSO at concentrations 1000 times greater than the nominal dosing concentration. Intermediate dilutions of PCDHs and DMSO were prepared in TCM just prior

to dosing cells. TNP(trinitrophenyl)-haptenated bovine serum albumin (TNP-BSA) was prepared according to the method of Garvy et al. (1977).

Culture Conditions for In Vitro Antibody Assay

Conditions for culturing chinook salmon splenocytes for *in vitro* antibody production were adapted from Arkoosh and Kaattari (1992). Spleen cell suspensions from individual salmon were prepared as described by Noguchi et al. (1997a). However, after the final washing the cells were resuspended in tissue culture medium (TCM) which contained RPMI-1640 supplemented with non-essential and essential amino acids, sodium pyruvate, L-glutamine, nucleosides (adenosine, uridine, cytidine, and guanine), 2-mercaptoethanol, gentamicin sulfate, and homologous chinook salmon plasma (1%). Preparation of homologous plasma was described previously (Noguchi et al., 1997a). Lymphocytes were enumerated by microscopic examination in a hemocytometer and viability was determined by trypan blue exclusion. The volume of cell suspension was adjusted to achieve a final cell concentration of 1×10^7 cells mL^{-1}.

Cell cultures were dosed with PCDHs by adding 25 µL of PCDH or DMSO solution (in TCM) and 1 µL of TNP-LPS to 474 µL of cell suspension. The suspension was mixed by aspirating it with a repeat-dispensing pipetter after which a 100 µL aliquot was dispensed into 4 wells of a flat-bottom 96-well microtiter plate. The final concentration of DMSO in PCDH and vehicle control cultures was 0.01%. Negative controls (cell suspension containing no TNP-LPS, PCDH or vehicle) and positive controls (cell suspensions containing TNP-LPS but no PCDH or vehicle) were assayed along with the treated cultures. All treatments were assayed in quadruplicate as a measure of the variability in responsiveness due to

methodological factors including differences in PCDH uptake and culture conditions among wells. The design does not allow assessment of variability associated with dose administration, *i.e.* pipetting error. The plates were covered with mylar plate sealers, placed in a sealed gas chamber that was purged with a 10% CO_2, 80% N_2, 10%O_2 gas mixture, and incubated at 17° C. Every 3 d, 10 µL of a nutrient supplement was added to each cell culture. After 12 d cultures were transferred to microcentrifuge tubes and centrifuged at 500xg for 10 min. The supernatants were removed, transferred to new microcentrifuge tubes and stored at -20° C.

ELISA for Anti-TNP Antibody Activity

TNP-specific antibodies were measured by indirect, antigen-excess ELISA. The TNP antigen was bound to wells of a round-bottom 96-well microtiter plate (Corning) by incubating 100 µL of TNP-BSA in carbonate buffer (147 ug TNP-BSA mL^{-1}) overnight at 4° C. The contents were removed by rapidly flicking the inverted plate. The plate was then washed 3x with a wash buffer (0.05% BSA and 0.1% Tween 20 in PBS). Wells were filled with a blocking buffer (3% BSA, 0.5% Tween 20 and 0.025% sodium azide in PBS) and incubated at 37° C for 1.5 hr. Plates were washed 3x then 50 µL of serum or culture supernatant was added to appropriate wells and incubated at 17° C for 1.5 hr. The serum or supernatants were removed and the plate was washed 3x, then 50 µL of Warr 1.14 antibody (1:4000 dilution) was added to each well. The plates were incubated at room temperature for 1 hr then washed 3x. Next, 50 µL of secondary antibody (HRP-conjugated goat anti-mouse Ig @ 1 µg mL^{-1}) was added to each well and incubated at room temperature for 30 min. The plates were washed 3x then 100 µL of HRP substrate (TMB + H_2O_2) was added to

each well and incubated at room temperature for 10 min to allow the color to develop. A Cayman microplate autoreader (Cayman Analytical, Ann Arbor, MI) was used to measure the optical density (OD) at 650 nm. Serum from TNP-KLH immunized rainbow trout (RBT), provided by Dr. Mary Arkoosh (National Marine Fisheries Service, Newport, OR), was used as a standard for TNP-specific antibody activity. Dilutions of RBT sera, culture supernatants, Warr 1.14 antibody and the secondary antibody were prepared in wash buffer.

Affinity ELISA

An ELISA technique described by Kaattari and Shapiro (1994) was used to characterize the anti-TNP binding affinity of antibodies in RBT sera and supernatants from chinook salmon splenocyte cultures. The assay is based on the principal that high antigen concentrations in the stationary phase (bound to the wells) are required for low affinity antibodies to bind whereas antibodies with high affinity can bind at low antigen concentrations. Thus, an affinity profile can be generated by culturing sera or supernatants in wells containing a range of antigen concentrations. The affinity of antibodies binding at each antigen concentration was determined by measuring the concentration of free antigen (TNP-lysine) that reduced binding to the stationary phase (plate) by 50%. Binding of high affinity antibodies to stationary antigen would be depressed by lower concentrations of free antigen than the binding of lower affinity antibodies.

Seven concentrations of TNP-BSA (0.037 - 147 μg/mL) were prepared in carbonate buffer and 100 μL of each concentration was added to all wells of one row of a round-bottom 96-well microtiter plate (Figure 15). The first row (A) received carbonate buffer containing no TNP-BSA. The plate was incubated overnight at 4° C. The plate was washed

and active sites were blocked with blocking buffer as previously described. Five concentrations of free TNP competitor (TNP-lysine) were prepared in wash buffer ($1x10^{-6}$ - $1x10^{-2}$ M) and 25 µL of each concentration was added to duplicate wells (Figure 15). Wells in columns 1 and 2 received 25 µL of wash buffer alone. All wells then received 25 µL of culture supernatant at a dilution which would generate 50% of maximun OD. The appropriate dilution was determined from prior analysis of supernatant titer. The plate was incubated at 17° C for 1.5 hr. The remaining steps, *i.e.* addition of Warr 1.14 antibody, secondary antibody, HRP substrate (TMB) and measurement at 650 nM, were the same as described for the TNP-specific ELISA. Apparent affinity constants (aK) were calculated from the following equation:

$$aK = \log (1/[H_{50}]) \qquad (1)$$

where:

$[H_{50}]$ = concentration of competitor (TNP-lysine) at 50% of max 650 nm OD calculated from competitive-inhibition curves.

Weighted average affinities (K_o) were calculated with equation 2.

$$K_o = \sum aK \times p \qquad (2)$$

where:

p = the proportion of total TNP-specific antibody with a give binding affinity.

Statistics

All statistical tests were performed using SYSTAT statistical software (Version 5.1), Evanston, IL. Effects of PCDHs on antibody production in splenocyte cultures from

individual fish were analyzed by one-way analysis of variance (ANOVA) and tukeys test was used to make pairwise-comparison of treatment means.

RESULTS

Optimizing ELISA Conditions

ELISA conditions were initially established for detecting TNP-specific antibodies in standard anti-sera, *i.e.* serum from TNP-KLH immunized rainbow trout (RBT). The RBT sera contained high anti-TNP antibody activity and could be detected by ELISA even at low concentrations of coating antigen (0.036 ug TNP-BSA mL^{-1}). Preliminary attempts to induce TNP-specific antibody production in splenocyte cultures resulted in low antibody activity; less than a 2 fold increase in antibody activity compared to unstimulated controls. Because of this low *in vitro* antibody production, ELISA conditions were reevaluated to determine whether they were suitable for detecting TNP-specific antibodies generated in culture supernatants. The concentration of coating antigen was assessed to determine whether concentrations of TNP-BSA that bind serum antibodies were equally effective at binding antibodies in culture supernatant. RBT sera and culture supernatants were assayed with varying concentrations of TNP-BSA antigen. At the antigen concentration used in the initial tests (0.036 ug TNP-BSA mL^{-1}) about 70% of the of the total TNP-specific antibody activity in RBT sera was detected, whereas, less than 5% of the antibody activity in culture supernatant was detectable (Figure 16). Much greater antigen concentrations were required for detecting maximal antibody activity in culture supernatants (147 ug TNP-BSA mL^{-1}) than in the RBT sera (0.58 ug TNP-BSA mL^{-1}). These greater antigen concentrations suggest that the antibodies produced *in vitro* had lower affinity for the TNP antigen that antibodies in the RBT sera.

Analysis of TNP-specific antibodies by affinity ELISA confirmed that antibodies detected in culture supernatants did, in fact, have lower affinities than antibodies in the RBT sera (Table 11). Apparent affinity constants (aK) were determined from competitive-inhibition curves for culture supernatant and RBT sera (Figure 17). The antibody affinity profiles indicate that while most of the TNP-specific antibody activity in supernatants was due to low affinity antibodies (aK 2.98 - 3.69) a small, but detectable, proportion of high affinity antibody (aK 6.3) was also present (Figure 18). Nevertheless, the weighed average affinity (K_o) of TNP-specific antibodies was less in culture supernatant (3.3) compared to RBT sera (6.3; Table 11).

Quantitation of TNP-Specific Antibody in Supernatants

Serum from TNP-KLH immunized RBT was used as a standard for quantitation of TNP-specific antibody activity in culture supernatants. Standard curves were generated by analyzing serial (2x) dilutions of RBT sera; from 1:2000 to 1:1,024,000. A representative standard curve is shown in Figure 19. The curves were consistently linear between 1:32,000 and 1:512,000 dilutions when the responses (ODs) were logit-transformed and the serum concentrations were log transformed. The OD at the least dilution within the linear range (1:32,000) was considered to be the max OD and it ranged from 0.9 to 1.1. The level of TNP-specific antibody activity in RBT sera was standardized by assigning a value of one unit of antibody activity to the serum concentration at ½ max OD. The antibody activity of undiluted RBT sera was then calculated by multiplying the dilution factor at ½ max OD by the activity at ½ max OD (1 unit). The average antibody activity in RBT sera from 11 standard curves was 1466 units of activity μL^{-1} serum (Table 12).

TNP-specific antibody activity of culture supernatants was calculated from RBT sera standard curves. The appropriate dilution of supernatants for quantitative ELISA was determine by screening samples in order to select a dilution that would result in an OD that was within the linear range of the standard curve. Since antibody activity was based on one dilution per sample, it was also important to demonstrate that response curves for supernatant antibodies were parallel to RBT standard curves. Departures from parallelism would indicate that the response is either attenuated or strengthened by confounding substances in the supernatant. Supernatant from two *in vitro* splenocyte cultures were titered and the response curves were compared to a RBT standard curve (Figure 19). Slopes of the logit-transformed response curves for culture supernatants (-1.98 and -2.14) were within the 95% confidence interval (Neter and Wasserman, 1974) for the slope of the RBT standard curve (-2.10). Thus, the RBT sera standard curve was appropriate for calculating antibody activity in the culture supernatants.

Effects of PCDH on In Vitro Antibody Production

Exposure of splenocyte cultures to TCDD resulted in a dose-related decrease in TNP-specific antibody production (Figure 20A). Statistically significant reductions in antibody activity were detected at concentrations of 3 and 30 nM TCDD. A downward trend in antibody activity was also observed in cultures exposed to increasing concentrations of PCB-126 (Figure 20B). Antibody activity was depressed by approximately 50% at the greatest concentration (300 nM PCB-126), however, this effect was not statistically significant at the 0.05 level because of variability within treatments. The response of splenocyte cultures to Aroclor 1254 exposure was less predictable (Figure 20C). Antibody activity was enhanced

at the greatest concentration, 9 μM Aroclor 1254, but the effect at lower concentrations was less certain.

Effects of TCDD and PCB-126 on antibody affinity were evaluated by affinity ELISA. TNP-specific antibody affinities were determined from cultures treated with 3 nM TCDD and 300 nM PCB-126. Supernatants from replicate culture wells were pooled in order to have sufficient sample volume to perform the assay. Antibody affinities were calculated from competitive-inhibition curves (Figure 21). The average affinity (K_o) of antibodies produced *in vitro* appeared to be unaffected by TCDD or PCB-126 (K_o 3.5 - 4.0; Table 13). However, close inspection of affinity profiles revealed that high affinity antibodies (aK 5.6 - 6.3) present in control cultures were absent in supernatant from TCDD-treated or PCB-126 treated cultures (Figure 22). Elimination of high affinity antibodies by treatment with 3 nM TCDD or 300 nM PCB-126 may have contributed to the lesser antibody activity observed in cultures exposed to TCDD and PCB-126 at those concentrations (Figures 20A&B).

DISCUSSION

Conditions are described for the *in vitro* induction of TNP-specific antibodies by chinook salmon splenocytes and for the detection of antibody activity by ELISA. With slight modifications, culture conditions that support *in vitro* AFC responses (Arkoosh and Kaattari, 1992) were also suitable for inducing antibody production *in vitro*. However, the detection of antibodies in culture supernatants by ELISA required considerably greater concentrations of coating antigen (TNP-BSA) than for the detection of standard antisera. Greater antigen concentrations were necessary because of the low affinity of TNP-specific antibodies produced *in vitro*. The difference between the affinity of antibodies in splenocyte cultures and

TNP-specific antibodies in standard antisera (produce in vivo), could be explained by affinity maturation.

Affinity maturation is a characteristic of the amnastic (memory) humoral response in which there is an increase in antibody affinity following secondary and subsequent exposure to antigen (Roitt et al., 1989). In mammals, the increase in affinity is accompanied by a switch in the predominant form (isotype) of antibody produced, from IgM to IgG (Roitt et al., 1989). Secondary exposure to antigen results in an increase in the affinity of IgG antibodies whereas the affinity of IgM antibodies remains unchanged. Fish possess a more restricted repertoire of antibody isotypes compared to mammals and the process of isotype switching does not appear to be a feature of the memory response in teleosts (Kaattari, 1992). The predominant form of serum antibodies in teleosts is an IgM-like tetrameric immunoglobulin (Kaattari and Piganelli, 1996). Until recently, there has been no evidence that affinity maturation occurs in fish (Arkoosh et al., 1991a). However, an affinity ELISA technique developed by mammalian immunologists (Nieto et al., 1984) and adapted for fish by Kaattari and Shapiro (1994) has enabled the detection of antibodies expressing a range of affinities for antigen. Using the affinity ELISA, Shapiro (1995) demonstrated that the rainbow trout receiving a single (priming) dose of antigen produced antibodies with lower affinity than rainbow trout that were exposed to antigen multiple times. In addition, there was a time-dependent increase in antibody affinity following a single immunization. Increases in antibody affinity are attributed to decreasing antigen concentrations over time. Only B cells with high affinity antigen receptor are capable of responding at low antigen concentrations, therefore, the proportion of high affinity antibody increases as antigen concentration diminishes (MacLennan and Gray, 1986). Thus, it should not be surprising that

in vitro immunization of splenocytes from naive chinook salmon induced production of predominantly low affinity antibodies, analogous the primary *in vivo* response. Antibodies with much greater affinity for TNP were detected in standard sera from rainbow trout that had received repeated immunizations. Multiple and prolonged exposure of rainbow trout to antigen (*in vivo*) would have provided the necessary conditions for affinity maturation to occur and could explain the predominance of high affinity antibody that was detected by affinity ELISA.

It should be noted that the segregation of antibodies into discrete affinity subclasses, as determined by affinity ELISA, is influenced by the design of the ELISA. The maximum number of antibody subclasses that can be detected is limited by the number of different coating antigen concentrations (TNP-BSA). In the present study seven concentrations of TNP-BSA were used, therefore, no more than seven antibody affinities could have been detected when, in fact, there may be a continuum of affinities. Thus, the affinity profiles generated by ELISA are estimations of the actual affinity distributions. The most useful information that can be derived from the affinity profiles is the range of antibody affinities and the weighted average affinity (K_o).

The ability to induce antigen-specific antibody synthesis *in vitro* combined with the quantitative ELISA provided a model system for assessing direct effects of PCBs and TCDD on humoral immunity in chinook salmon. The antibody response integrates all aspects of humoral immunity including, antigen detection, interactions between B cells and accessory cells, clonal proliferation, terminal B-cell differentiation and antibody synthesis. The proliferative response of chinook salmon splenocytes was previously shown to be affected by direct exposure to TCDD (Noguchi et al., 1997b) and this study demonstrated that TCDD

also suppresses antibody production. Based on these results it can be concluded that TCDD can alter humoral immune responses in chinook salmon. Concentrations of TCDD that affected antibody synthesis and lymphocyte proliferation (3 and 30 nM) were comparable to concentrations that suppress the *in vitro* AFC response in murine splenocytes (IC_{50} 7 nM; Harper et al., 1995). This indicates that the intrinsic sensitivity of the humoral immune response in chinook salmon to TCDD is similar to that of B6C3F1 mice, one of the more sensitive mammalian models for TCDD-induced immunotoxicity. PCB-126 exhibited immunosuppressive tendencies, although great within-treatment variability diminished the statistical significance of these effects. Nevertheless, an average reduction in antibody activity of about 50%, observed at the greatest concentration of PCB 126 (300 nM), may be biologically significant. PCB-126 also suppresses lymphocyte proliferation (Noguchi et al., 1997b), although the potency was less than that of TCDD (toxic equivalency factor 0.0011). Similarly, greater concentrations of PCB 126 were needed to depress antibody production compared to TCDD. In contrast, the sensitivity of murine humoral responses to PCB 126 (EC_{50} 9.1 nM) and TCDD (EC_{50} 7.0 nM) are similar (Harper et al., 1995). Structure-activity relationships for immunomodulation as well as other effects such as enzyme induction (Janz and Metcalfe, 1991; Newsted et al., 1995) and early life-stage mortality (Walker and Peterson, 1991) indicate that planar PCB congeners are less potent at inducing dioxin-like effects in fish than in mammals.

Aroclor 1254 suppresses *in vitro* lympho-proliferative responses but only at concentrations that are also cytotoxic (45 and 90 μM; Noguchi et al., 1997b). In the present study, Aroclor 1254 was tested at non-cytotoxic concentrations (0.45 to 9 μM) and no significant depression in antibody activity was detected. However, at lesser concentrations

Aroclor 1254 can enhance antibody production (Figure 20C). Up-regulation of B-cell activity has also been observed in fish exposed *in vivo* to PCBs (Thuvander et al., 1993; Rice and Schlenk, 1995) and to complex mixtures of environmental contaminants containing PCBs (Faisal et al., 1991a). However, in some studies PCBs have been ineffective at modulating immune responses (Cleland and Sonstegard, 1987; Cleland et al., 1988a) while in others PCBs were immunosuppressive (Arkoosh et al., 1994a). Whether PCBs stimulate, suppress or have no effect on the piscine immune system may well be related to the concentration of PCBs that accumulates in target cells. Aroclor 1254 appears to have a rather steep dose-response relationship in terms of direct effects on humoral immunity (*in vitro*), because there was only a 5-fold difference between concentrations that were cytotoxic and concentrations that were immunostimulatory. This type of response does not allow for discrimination of effects beyond the limited range of concentrations that either stimulate or kill susceptible cells and could lead to the apparent contradictory results mentioned above. Interpretation of results from *in vivo* exposure studies is further complicated by the fact that tissue or cellular concentrations of PCBs are rarely reported and differences in exposure method, *e.g.* IP injection or dietary exposure, preclude the determination of potency based solely on exposure.

The suppressive effects of TCDD and PCB-126 on antibody production were accompanied by the elimination of antibodies with high affinity for the TNP antigen. Because B cells express antigen receptors that are analogues of the antibody they will produce, high affinity antibodies are produced by B cells with high affinity antigen receptors. B cells expressing high affinity receptors would be more sensitive to antigen-induced activation than cells with low affinity receptors. The heightened level of activation attained by clones expressing high affinity receptor may have made these cells more susceptible to TCDD and

PCB-126. There are two lines of evidence which indicate that the effectiveness of TCDD at inducing biochemical or immunomodulatory effects is dependent on the activation state of the lymphocytes. Induction of 7-ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) synthesis are measures of the biochemical activity of TCDD and other AhR agonist. TCDD induces EROD synthesis in mitogen-stimulated human lymphocytes but not in unstimulated lymphocytes (Wood et al., 1993). TCDD also induces AHH activity in a human lymphoblastoid cell line following mitogen stimulation (Waithe et al., 1991). Similarly, high density (resting) B cells are refractory to TCDD, whereas, TCDD suppressed both background proliferation and IgM secretion by low density (activated) B cells (Morris et al., 1993; Wood et al., 1993). Thus, the state of lymphocyte activity may be an important factor in regulating the efficacy of TCDD and may render B cells expressing high affinity antigen receptors particularly susceptible to TCDD and other AhR agonists.

If B cells expressing high affinity receptors are in fact more sensitive to ArR agonists then secondary humoral responses should be more sensitive than the primary response. As a result of affinity maturation and clonal proliferation (Kaattari, 1992), B-cell clones expressing high affinity antigen receptors would be expected to constitute a greater proportion of the responding cells during the memory response. Thus, preferential suppression of high affinity clones would diminish a greater proportion of responding cells during the memory response than during the primary response. Secondary AFC responses have been reported to be more sensitive than primary responses in chinook salmon treated with Aroclor 1254 or dimethylbenz[a]anthracene (Arkoosh et al., 1994a) and in chinook salmon retrieved from a PCB/PAH (polycyclic aromatic hydrocarbon)-contaminated estuary in Puget Sound (Arkoosh et al., 1991). The mechanism underlying chemical-induced

immunosuppression of secondary humoral responses has yet to be elucidated, however, results from the present study indicate that greater sensitivity of B cells expressing high affinity antigen receptor may contribute to a diminished memory response.

In conclusion, *in vitro* induction of antibody production combined with the detection of antibody activity and affinity by ELISA provided a system for assessing the potency with which PCBs and TCDD modulate humoral immunity in chinook salmon and for revealing possible mechanisms of action.

Table 11. Weighted-average affinity constants (K_o) for TNP-specific antibodies in supernatants from TNP-LPS stimulated chinook salmon splenocyte cultures and serum from TNP-KLH immunized rainbow trout. (aK - apparent affinity constant; p- proportion of total TNP-specific antibody with a given aK)

Culture Supernatant				Rainbow Trout Sera			
TNP-BSA ($\mu g\ mL^{-1}$)	aK	p	aK * p	TNP-BSA ($\mu g\ mL^{-1}$)	aK	p	aK * p
0.576	6.33	0.057	0.36	0.009	7.48	0.0909	0.68
2.3	3.69	0.305	1.13	0.036	7.31	0.195	1.43
9.2	3.15	0.366	1.16	0.144	6.69	0.26	1.74
36.9	3.18	0.128	0.41	0.576	5.89	0.258	1.52
147	2.98	0.143	0.43	2.3	5.18	0.178	0.92
Weighted-Average Affinity (K_o):			3.3				6.3

Table 12. TNP-specific antibody activity of serum from TNP-KLH immunized rainbow trout. Values from different 11 standard curves.

Plate	½ Max OD	Logit at ½ Max OD	µL Serum per well at ½ Max OD	Units of Anti-TNP Antibody Activity per µL Serum
1	0.560	-1.444	0.00071	1418
2	0.565	-1.745	0.00068	1465
3	0.495	-2.019	0.00068	1471
4	0.528	-1.779	0.00065	1536
5	0.473	-1.337	0.00067	1482
6	0.453	-1.786	0.00068	1470
7	0.467	-1.670	0.00066	1521
8	0.447	-1.939	0.00070	1419
9	0.497	-1.778	0.00067	1501
10	0.479	-2.074	0.00071	1404
11	0.492	-1.857	0.00070	1433
			Mean	1466
			SD	43
			CV	0.03

Table 13. Weighted-average affinity constants for TNP-specific antibodies in supernatants from chinook salmon splenocyte cultures that were stimulated with TNP-LPS and dosed with TCDD or PCB 126. (aK - apparent affinity constant; p- proportion of total TNP-specific antibody with a given aK)

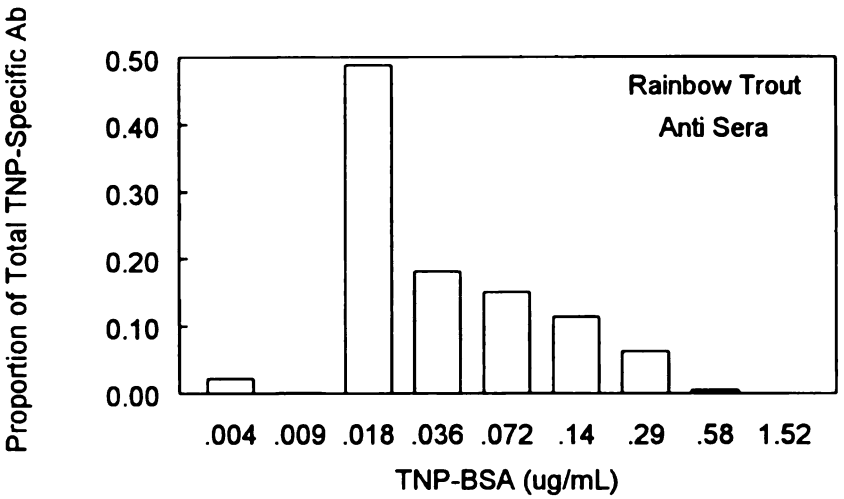
TNP-BSA	Control			Vehicle			3 nM TCDD			300 nM PCB 126		
($\mu g\ mL^{-1}$)	aK	p	aK * p	aK	p	aK * p	aK	p	aK * p	aK	p	aK * p
0.57	6.33	0.092	0.58	5.62	0.036	0.20	4.35	0.08	0.35	4.33	0.075	0.32
2.3	4.02	0.363	1.46	3.66	0.38	1.39	3.69	0.399	1.47	3.85	0.374	1.44
9.2	3.61	0.234	0.84	3.37	0.322	1.08	3.43	0.336	1.15	3.52	0.298	1.05
36.9	3.54	0.087	0.31	3.25	0.133	0.43	3.41	0.121	0.41	3.49	0.125	0.44
147	3.73	0.229	0.85	3.25	0.112	0.36	3.27	0.073	0.24	3.36	0.11	0.37
Weighted-Average Affinity:			4.0			3.5			3.6			3.6

TNP-LYS (M) :		0		$5x10^{-7}$		$5x10^{-6}$		$5x10^{-5}$		$5x10^{-4}$		$5x10^{-3}$	
TNP-BSA ug/mL		1	2	3	4	5	6	7	8	9	10	11	12
0	A												
0.036	B												
0.14	C												
0.57	D												
2.3	E												
9.2	F												
36.9	G												
147	H												

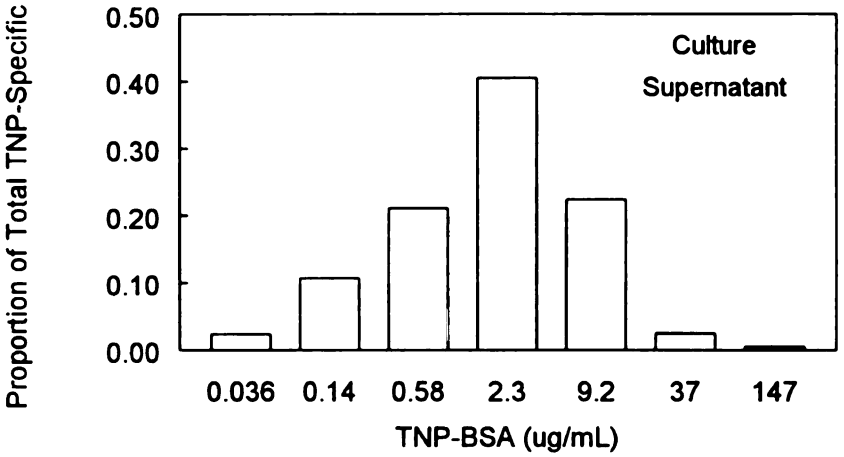
Figure 15. Layout for affinity ELISA. Each supernatant sample was analyzed on an individual ELISA plate. Duplicate wells are reserved for each combination of coating antigen (TNP-BSA) concentration and competitor (TNP-lysine) concentration. Shading represents the expected magnitude of antibody bound to stationary antigen (TNP-BSA); ■-greatest concentration of bound antibody, □-least concentration of bound antibody.

Figure 16. Affinity ELISA: Proportion of total TNP-specific antibody bound at different concentrations of TNP-BSA coating antigen; A) serum from TNP-KLH immunized rainbow trout, B) supernatant from TNP-LPS stimulated splenocyte cultures, C) cumulative proportion of TNP-specific antibody bound at different TNP-BSA antigen concentrations (● rainbow trout serum, ▲ splenocyte culture supernatant).

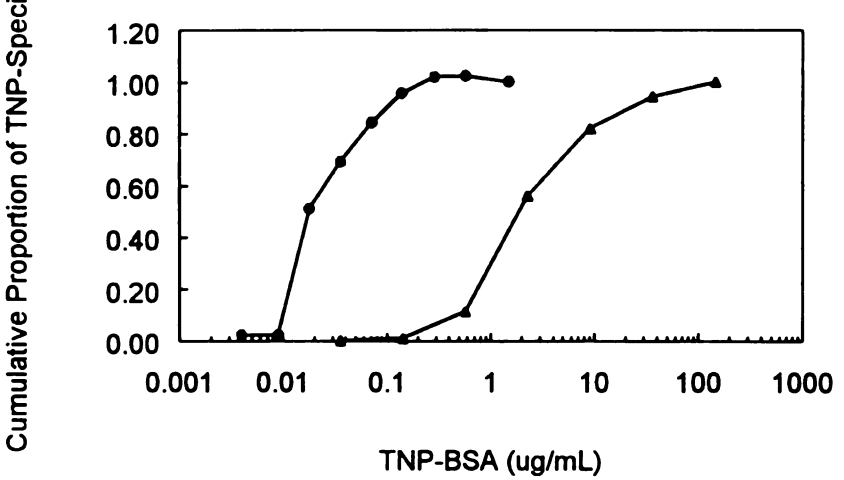
(A)


Proportion of Total TNP-Specific Ab
0.50
0.40
0.30
0.20
0.10
0.00
Rainbow Trout
Anti Sera
.004 .009 .018 .036 .072 .14 .29 .58 1.52
TNP-BSA (ug/mL)

(B)


Proportion of Total TNP-Specific Ab
0.50
0.40
0.30
0.20
0.10
0.00
Culture
Supernatant
0.036 0.14 0.58 2.3 9.2 37 147
TNP-BSA (ug/mL)

(C)


Cumulative Proportion of TNP-Specific Ab
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.001 0.01 0.1 1 10 100 1000
TNP-BSA (ug/mL)

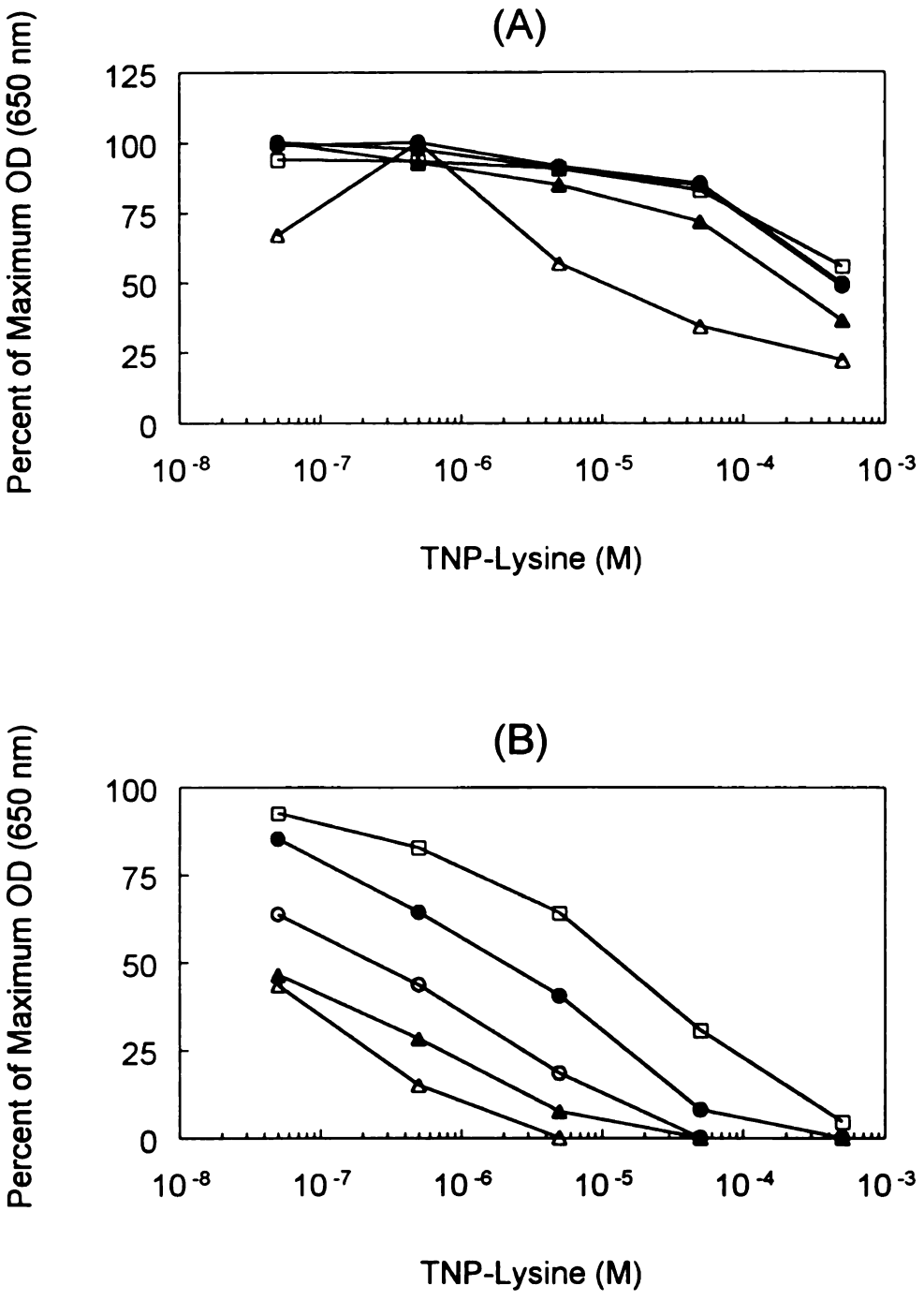


Figure 17. Competitive-inhibition curves from affinity ELISA with A) supernatant from TNP-LPS stimulated chinook salmon splenocyte cultures and B) serum from TNP-KLH immunized rainbow trout.

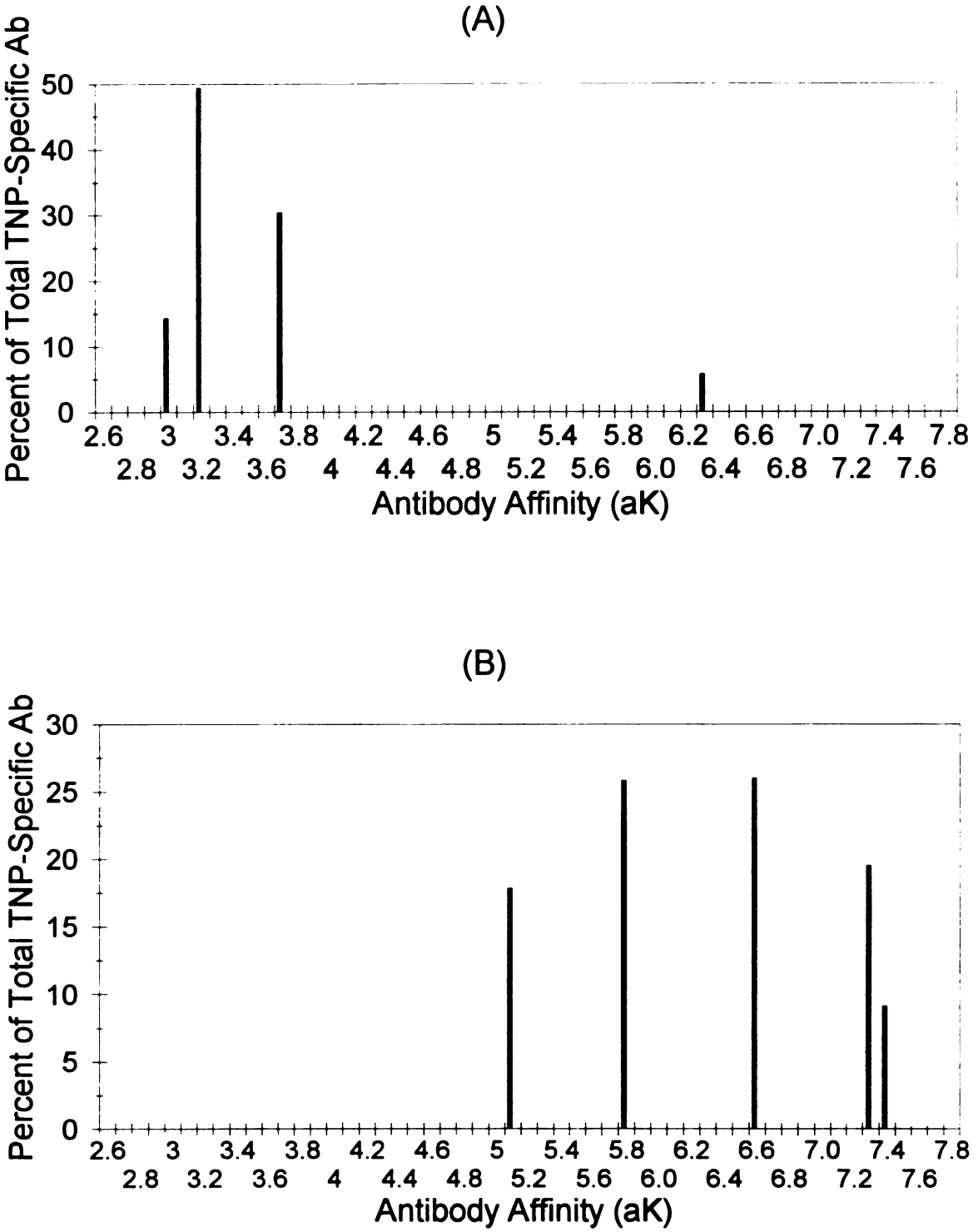


Figure 18. Affinity distribution of TNP-specific antibodies in A) supernatant from TNP-LPS stimulated chinook salmon splenocyte cultures and B) serum from TNP-KLH immunized rainbow trout.

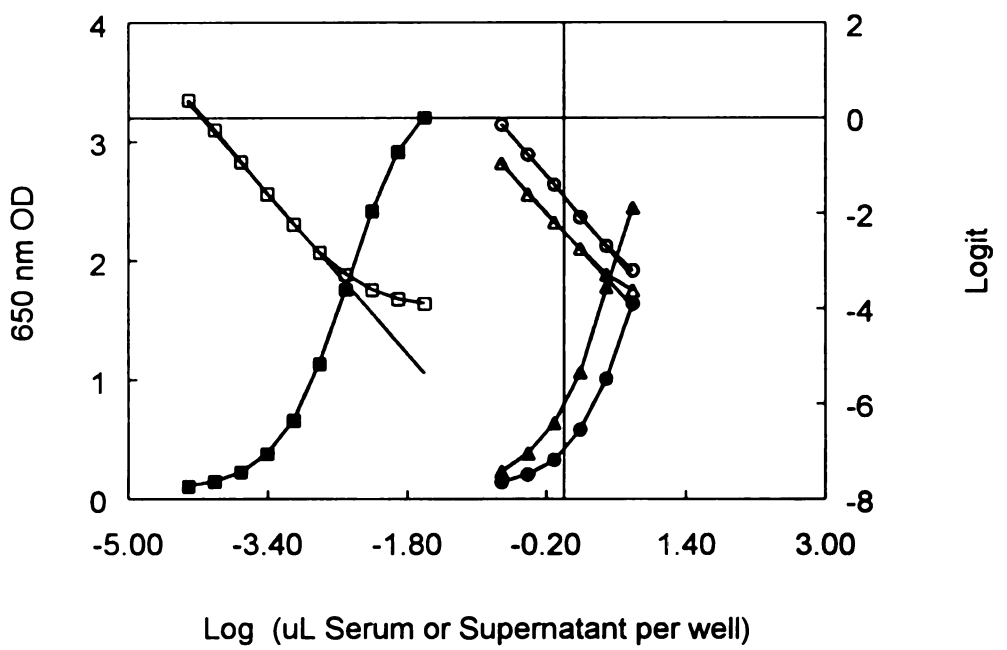


Figure 19. A representative standard curve for TNP-specific antibody activity in standard rainbow trout serum (■ 650 nm OD; ❑ logit 650 nm OD), and dose-response curves for supernatants from two TNP-LPS stimulated splenocyte cultures (● & ▲ - 650 nm OD, ○ & Δ logit transformed 650 nm OD).

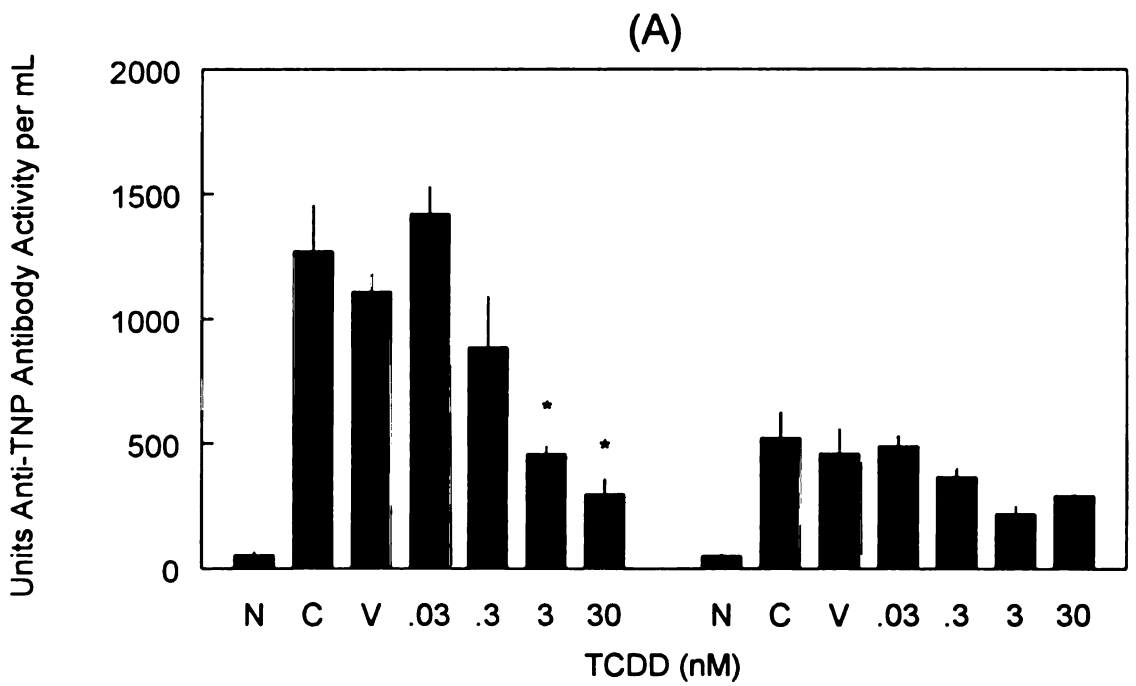


Figure 20. Direct effects of TCDD (A), PCB-126 (B) and Aroclor 1254 (C) on *in vitro* antibody production by chinook salmon splenocytes. Values are means ±SE for the 4 replicate wells. Responses from *in vitro* cultures from 2 fish are shown for each compound. Asterisk denotes treatments that are significantly different than vehicle control ($\alpha = 0.05$). N - nonstimulated control, C - TNP-LPS stimulated control, V - vehicle control.

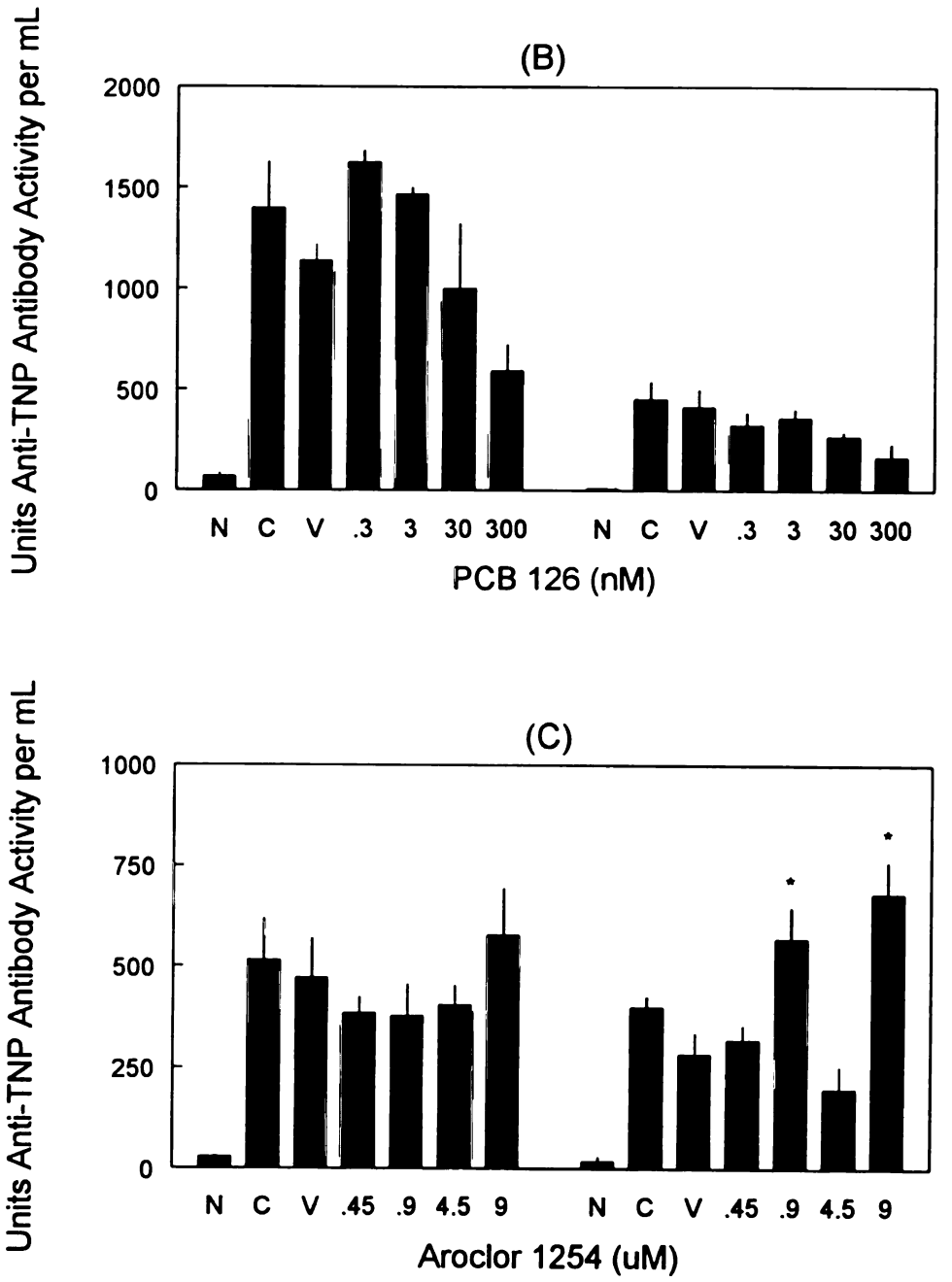
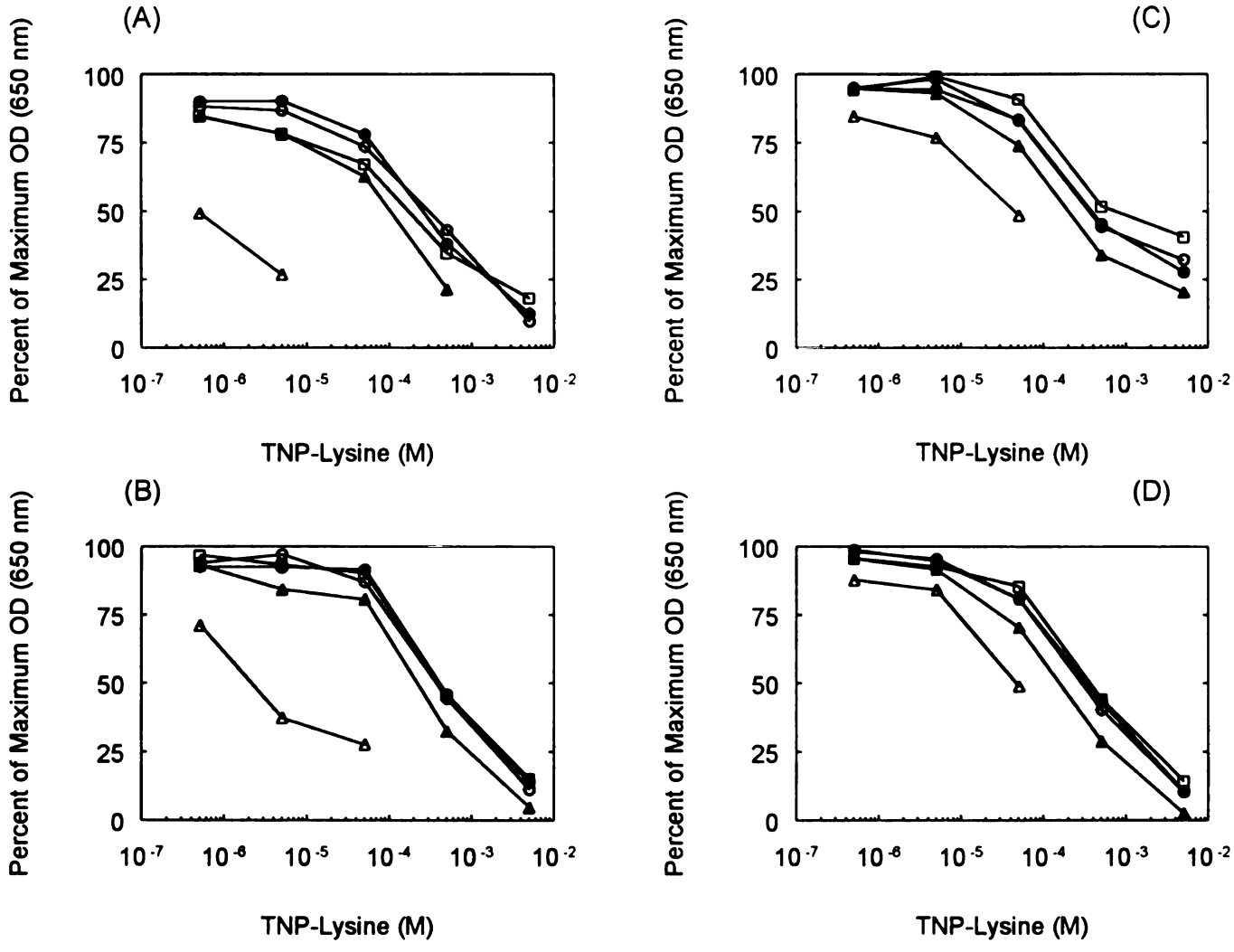


Figure 20 (continued).

Figure 21. Competitive-inhibition curves from affinity ELISAs with supernatants from chinook salmon splenocyte cultures treated with TCDD or PCB-126; A) TNP-LPS stimulated controls B) vehicle control C) 3 nM TCDD and D) 300 nM PCB-126.


(A)
(B)
(C)
(D)
Percent of Maximum OD (650 nm)
100
75
50
25
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
TNP-Lysine (M)

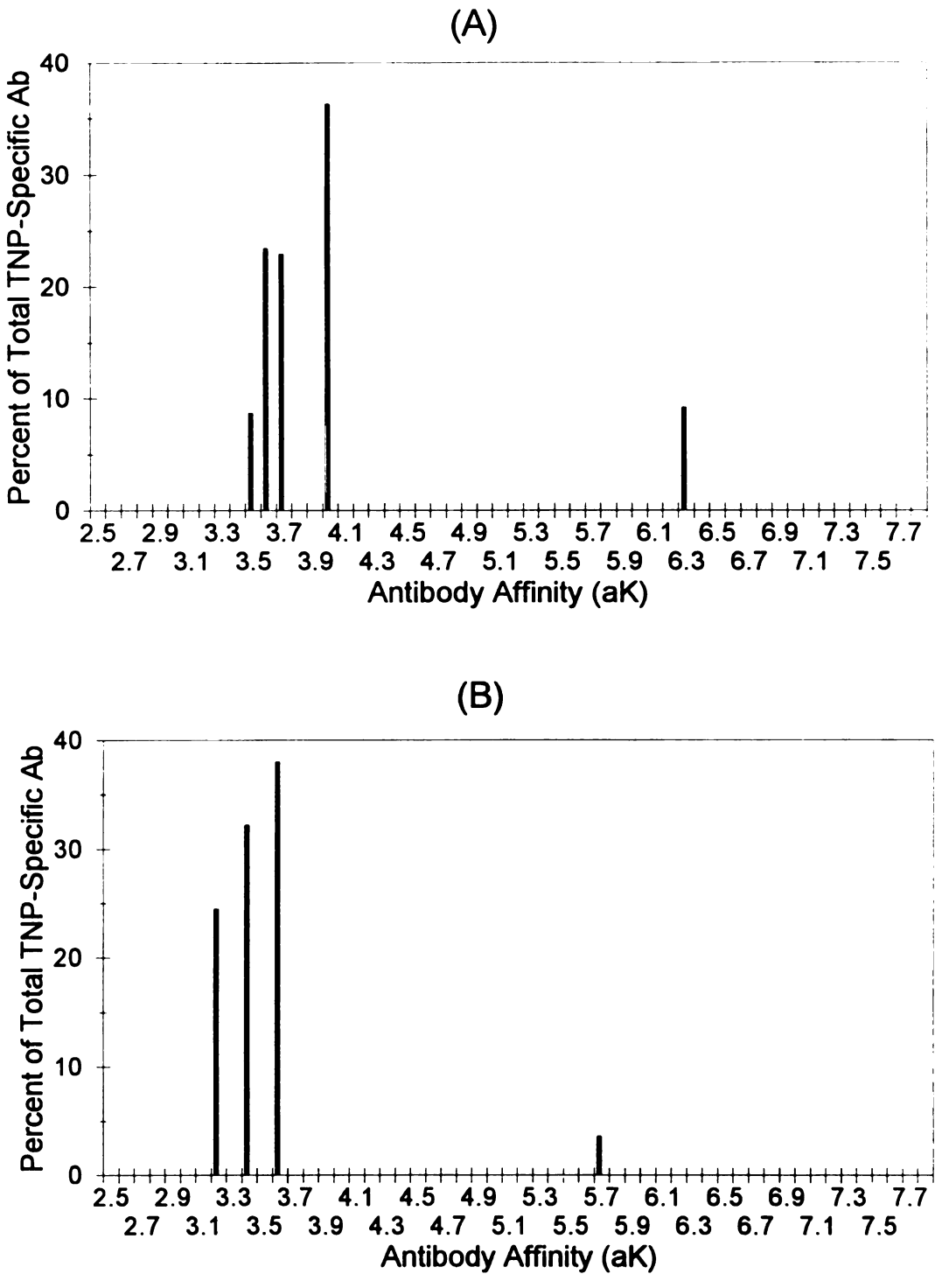


Figure 22. Affinity distribution of TNP-specific antibodies in A) TNP-LPS stimulated controls B) vehicle control C) 3 nM TCDD and D) 300 nM PCB-126.

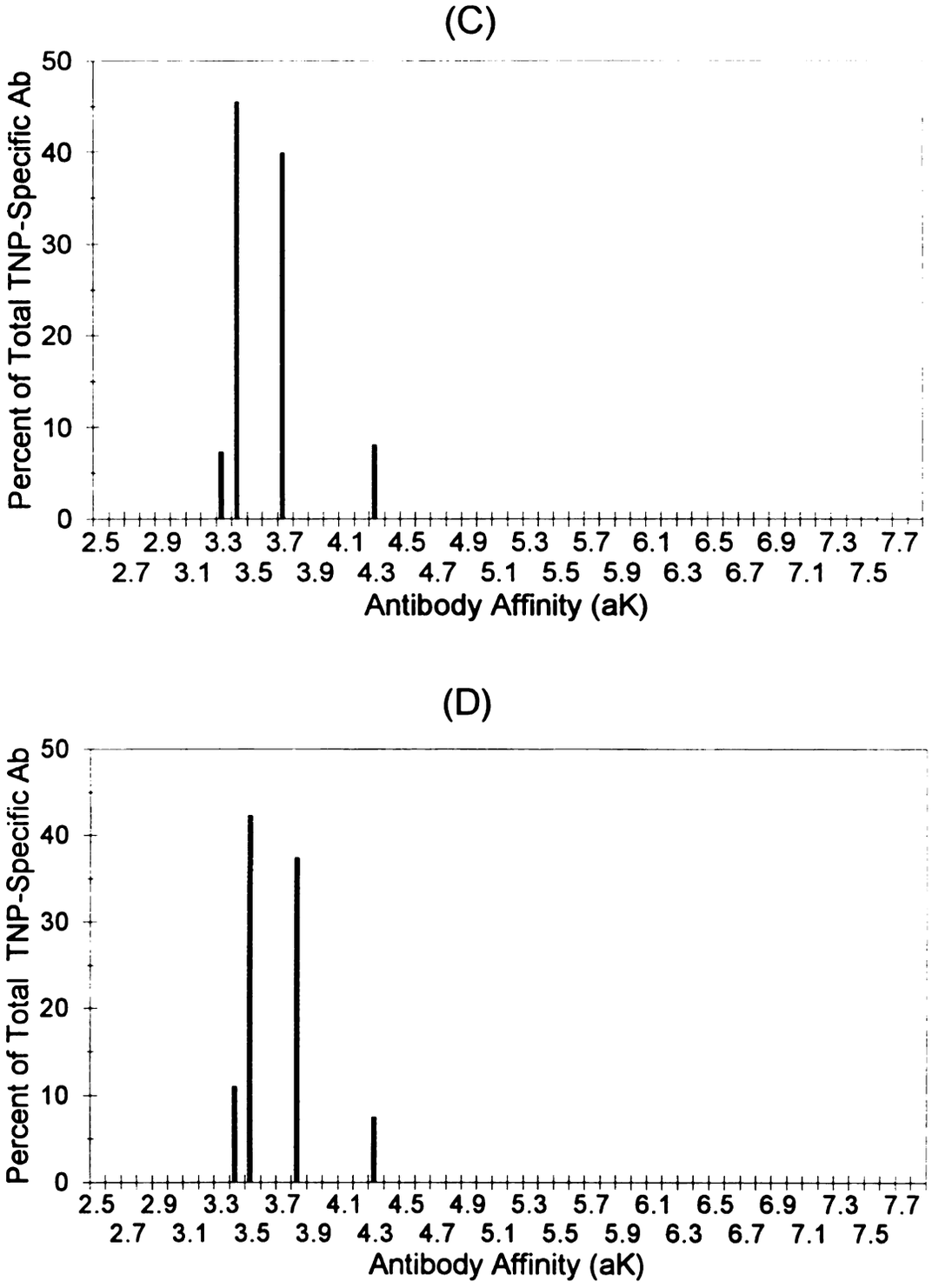


Figure 22 (continued).

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