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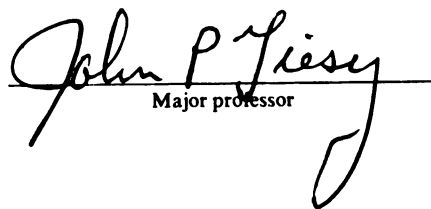
APPLICATION OF MECHANISM-SPECIFIC IN VITRO
BIOASSAYS TO ADDRESS QUESTIONS IN
ENVIRONMENTAL TOXICOLOGY

presented by

Daniel Lawrence Villeneuve

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of the requirements for

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Major professor

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APPLICATION OF MECHANISM-SPECIFIC *IN VITRO* BIOASSAYS TO ADDRESS
QUESTIONS IN ENVIRONMENTAL TOXICOLOGY

By

Daniel Lawrence Villeneuve

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Submitted to
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ABSTRACT

APPLICATION OF MECHANISM-SPECIFIC *IN VITRO* BIOASSAYS TO ADDRESS QUESTIONS IN ENVIRONMENTAL TOXICOLOGY

By

Daniel Lawrence Villeneuve

This dissertation presents four studies centered around the application of mechanism-specific *in vitro* bioassays in the field of environmental toxicology. The first study used *in vitro* bioassays to screen and rank 11 chemicals based on their affinity for the estrogen receptor (ER) and ability to elicit an estrogenic response *in vitro*. Six of the eleven chemicals tested were able to displace tritiated 17 β -estradiol from the ER. Their rank order of affinity was 17 β -estradiol (E2) > coumestrol > 17 β -ethynyl estradiol (EE2) > nonylphenol (NP) > octylphenol (OP) > bisphenol A (BPA). The rank order of potency for inducing reporter gene expression in the MCF-7-luc *in vitro* bioassay was E2 > EE2 > NP > OP > coumestrol > atrazine > BPA. ER binding and MCF-7-luc gene expression results were compared to generate hypotheses regarding potential mechanisms of action for the compounds analyzed. The second study focused on the development and characterization of a recombinant rainbow trout cell line (RLT 2.0)-based assay for assessing dioxin-like potency. Methods were adapted for a 96-well microplate format, and assay specific relative potencies (REPs) were derived for a number of halogenated aromatic hydrocarbons. Overall, the rank order of RLT 2.0-derived REPs was comparable to those generated based on other fish and mammalian bioassays, both *in vitro* and *in vivo*. A sensitivity analysis was also conducted to estimate the uncertainty associated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQ) calculated using

the REPs generated. Result indicated that variability in the RLT 2.0-derived REPs could yield up to 10-fold uncertainty in RLT 2.0-based TEQ estimates. The third study focused on the current debate surrounding the derivation, use, and misuse of *in vitro* bioassay-based REP estimates. A systematic approach for deriving REP estimates from non-ideal *in vitro* bioassay results was presented and demonstrated using example data sets. The final study was designed to examine the relevance of current *in vitro* models for predicting estrogenic potencies in fish. Sexually mature male common carp (*Cyprinus carpio*) were exposed to 4-nonylphenol. Potential indirect mechanisms of action involving the modulation of plasma steroid concentrations were examined. The study detected no treatment related increases in concentrations of plasma E2, testosterone, or vitellogenin, despite measurable levels of NP in the plasma and tissues of exposed carp. Unexpectedly high variability among fish, and plasma E2 and VTG concentrations below method detection limits limited the ability to resolve effects, however. The lack of a detectable estrogenic effect *in vivo* hindered the ability to calibrate the known *in vitro* potency of NP to its potency for producing estrogenic effects in sexually mature male carp. The lack of estrogenic response also raised questions regarding the utility of estimating plasma or tissue concentrations of 17 β -estradiol equivalents as a means of predicting the potential for estrogenic effects *in vivo*. Overall, the studies described in this dissertation exemplify research which is being done to develop and establish the utility of mechanism-specific *in vitro* bioassays as tools for environmental toxicology research and risk characterization.

Dedicated to my parents, Kathy and Larry Villeneuve, for a lifetime of unconditional support and encouragement.

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INTRODUCTION

There are currently over 100,000 chemicals in commerce (Trapp and Matthies 1998). Over 1000 of these have annual productions exceeding 1000 tons (Trapp and Matthies 1998). Hundreds of new chemicals enter the market each year (Zeeman et al. 1995, Trapp and Matthies 1998). Of these chemicals, a portion are released to the environment either directly (i.e. pesticides) or indirectly as byproducts of their use, accidents, etc. (i.e. automobile emissions, industrial effluents, oil spills, etc.). Once in the environment additional compounds may be formed as environmental processes such as photolysis, hydrolysis, oxidation, reduction, and biotransformation, transform parent compounds. Some compounds released into or formed in the environment have the potential to cause adverse effects in exposed organisms. In some cases, obvious adverse effects, such as acute toxicity, may occur. In other cases, effects may be subtle, but just as detrimental to the long term survival of exposed populations of organisms and the function of the ecosystems they are part of (Carson 1962). Eggshell thinning in birds exposed to DDT was a classic example (Carson 1962, Bowerman et al. 1995). In order to help avoid environmental tragedies, there is clearly a need to develop rapid, cost effective, and reliable methods to screen chemicals for their potential to elicit adverse biological effects, whether obvious or subtle. Furthermore, there is need to monitor and characterize the potential hazards posed by chemicals in the forms and complex mixtures in which they are found in the environment.

In vitro bioassays are ideally suited to this role. Field and laboratory studies with whole animals are critical for linking exposure to biologically relevant effects. They are,

however, impractical for routine, high throughput, screening of individual compounds or environmental samples. Procedurally, *in vitro* bioassays are typically performed more quickly and at significantly less cost than *in vivo* studies. Use of simplified biological systems circumvents much of the inter-individual, seasonal, and temporal variability which can confound interpretation of *in vivo* responses. Additionally, *in vitro* bioassays avoid many of the complex socio-political and ethical issues associated with whole animal studies (Stokes and Marafante 1998). Thus, although they are not a replacement for *in vivo* studies, *in vitro* bioassays are more amenable for routine use than *in vivo* assays.

In vitro bioassays also overcome some of the key limitations of instrumental analysis. While instrumental analyses are essential for the identification and quantitation of compounds in a sample, they provide no information regarding the capacity and potency of those compounds to elicit a biological effect. *In vitro* bioassays measure mechanistically-based biological responses. As a result, they can provide information regarding the biological relevance of a sample. *In vitro* bioassays provide an integrated measure of the combined potency of all compounds in a sample. This is a practical advantage, since instrumental analysis of complex mixtures can be very expensive, difficult, and time consuming. Additionally, from a functional stand-point, it means that *in vitro* bioassays can account for both compounds for which there are no analytical methods available, and potential additive and non-additive interactions between compounds. When the biological response being measured is highly sensitive, *in vitro* bioassays can also account for compounds which can exert a biological effect at concentrations below analytical detection limits. As a result, *in vitro* bioassays can serve

as simple, rapid, and sensitive tools for detecting the presence and mutual interactions of chemicals which function through a specific mode of action. *In vitro* bioassays applied in an iterative fashion along with successive chemical fractionation and instrumental analysis can be used to identify putative causative agents in complex mixtures. Thus, *in vitro* bioassays provide information which can complement instrumental analyses.

In recent years, a number of factors have spurred the development of *in vitro* bioassays as tools for addressing questions in environmental toxicology. Developments in molecular and cellular biology have led to significant advances in the understanding of molecular and cellular mechanisms of toxicity (Stokes and Marafante 1998). These have been paralleled by technological advances in tissue culture, genetic engineering, and automated testing equipment (Stokes and Marafante 1998). The development of *in vitro* bioassays has also been stimulated by public concern about the use of animals for testing (Stokes and Marafante 1998). In 1993, the U.S. National Institute of Environmental Health Sciences was directed, by law, to develop alternative methods that can reduce or eliminate the use of animals in acute and chronic safety testing (U.S. Code 1993). Thus, there appears to be an important and increasing role for *in vitro* bioassays to play in the field of environmental toxicology.

This dissertation presents research which centered around the application of mechanism-specific *in vitro* bioassays to address questions in environmental toxicology. In particular, it focuses on *in vitro* bioassays developed for the assessment of dioxin-like and estrogenic effects.

Chapter 1 provides an overview of the potential uses of *in vitro* bioassays in environmental toxicology, using the issue of environmental estrogens as an example. A

mechanistic basis for the development of *in vitro* bioassays to characterize estrogenic effects is presented. The advantages and disadvantages of a variety of *in vitro* testing methodologies are discussed. Experimental results presented in chapter 1 provide examples of the application of *in vitro* bioassays to screen chemicals for their potential to elicit a mechanism-specific biological response, rank them based on their potency to produce that response, and generate hypotheses regarding their potential mechanisms of action. Furthermore, estrogenic potencies reported in chapter 1 served as the basis for mass balance analyses conducted in later studies aimed at the identification and characterization of xenoestrogens in various environmental samples (Appendix A).

Chapter 2 presents methods for assessing dioxin-like potency using recombinant rainbow trout hepatoma cells (RLT 2.0). It addresses several important issues in *in vitro* bioassay development and application. Assay specific relative potencies, needed for mass balance analyses, were derived and reported for a number of important dioxin, furan, and polychlorinated biphenyl (PCB) congeners. The issue of predictive relevance was addressed both in terms of species specificity and calibration of *in vitro* and *in vivo* potencies by comparing RLT 2.0-derived relative potencies to other fish and mammalian bioassay-derived values. Finally, the relative potencies derived were used to calculate 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQ) for representative environmental samples and a sensitivity analysis was performed to evaluate the range of uncertainty in the TEQ value resulting from uncertainties in the congener specific relative potency estimates. Such analyses are important for mass balance applications in which a researcher must determine what magnitude of difference between bioassay derived toxic equivalents and instrumentally based equivalents is significant.

Chapter 3 presents a standardized procedure for deriving and interpreting relative potency estimates based on *in vitro* bioassay results. Relative potency estimates are widely used in environmental toxicology for ranking and comparing the potencies of chemicals and environmental samples. Currently, however, there is no consensus method for the derivation of relative potency estimates. Furthermore, such estimates are based on a number of assumptions which are often both violated and ignored. This chapter discusses the assumptions involved, the uncertainties that are generated when assumptions are violated, and proposes methods for addressing these issues when deriving estimates and presenting results.

Chapter 4 revisits the issue of environmental estrogens. Although the research presented is primarily *in vivo* in nature, it is closely tied to the application of *in vitro* bioassay methods in environmental toxicology. *In vitro* bioassays utilize vastly simplified biological systems. In order to be useful, *in vitro* bioassay results must be rigorously calibrated with *in vivo* endpoints of toxicological relevance and be shown to be predictive. The extent to which an *in vitro* assay can accurately model or predict the *in vivo* potency of a chemical or environmental sample is directly dependent on the relevance of the mechanism of action modeled for mediating the *in vivo* effects. For example, an *in vitro* assay which examines the ability of a compound or sample to elicit estrogen response element (ERE)-mediated gene expression would not detect compounds which exert estrogenic responses *in vivo* by acting as an anti-androgen, or by altering endogenous estradiol levels through effects on the hypothalamus or pituitary. The study presented in chapter 4 was designed to investigate whether 4-nonylphenol was able to elicit estrogenic effects in fish through an indirect mechanism which nearly all current *in*

in vitro assays for estrogenic activity would be unable to model. Similar studies, aimed at establishing the predictive relevance of *in vitro* bioassay responses are critical for enhancing and increasing the utility of *in vitro* assays in environmental research, monitoring, and risk assessment.

Studies presented in this dissertation, and referenced in Appendix A provide examples of the variety of applications for *in vitro* bioassays in the field of environmental toxicology. Additionally, they demonstrate the type of research and development required to fully develop their potential as research, monitoring, and risk assessment tools. Although it does not provide all the answers or address all the issues, the research presented in this dissertation should help facilitate and enhance the applicability existing *in vitro* bioassay methods and the interpretation of their results for addressing questions in environmental toxicology.

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Chapter 1

INTERACTIONS BETWEEN ENVIRONMENTAL XENOBIOTICS AND ESTROGEN RECEPTOR-MEDIATED RESPONSES

Daniel L. Villeneuve^{*}, Alan L. Blankenship, John P. Giesy.

**Dept. of Zoology, Pesticide Research Center, and Institute for Environmental
Toxicology, Michigan State University.**

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INTRODUCTION

Environmental Estrogens

Endocrine "disruption" by environmental contaminants has become a cause for concern among scientists, environmental advocates and politicians alike (1-3). A number of compounds released into the environment by human activities can modulate endogenous hormone activities and have been termed endocrine-disrupting compounds (EDCs) (3-5). It has been hypothesized that such compounds may elicit a variety of adverse effects in both humans and wildlife including promotion of hormone-dependent cancers, reproductive tract disorders, and a reduction in reproductive fitness (6).

The neuroendocrine system is a primary mechanism by which organisms maintain homeostasis (7). Thus, generalized adaptations to stress, which allow organisms to resist perturbations from normal homeostatic ranges, typically involve a variety of endocrine and physiological responses (8). As a result, any stressor could be loosely defined as an "endocrine disruptor". Furthermore, there are a number of receptor-mediated hormonal responses to chemicals. These include xenobiotic effects on thyroid hormone receptor (9), epidermal growth factor (EGF) receptor (10), aryl hydrocarbon receptor (AhR), as well as the estrogen and androgen receptor (ER and AR) mediated mechanisms. In this chapter, we will restrict our discussion to direct-acting estrogenic and antiestrogenic compounds. These will be defined as those compounds that bind competitively to the estrogen receptor (ER) and cause or inhibit estrogen-like responses *in vitro* or *in vivo*. Although our discussion focuses on estrogen agonists and antagonists, compounds that can cause tissue-level responses without ER binding are also covered.

Table 1. *Compounds implicated as xenoestrogens or antiestrogens*

Compounds	Reference
Organochlorine Pesticides	
DDT/DDE	32,33
toxaphene	34
dieldrin	34
chlorodecone	35
Polychlorinated biphenyls (PCBs)	36-38
Polycyclic aromatic hydrocarbons (PAHs)	39
Polychlorinated dibenzo dioxins (PCDDs)	40,41
Plasticizers (e.g. Bisphenol A)	42
Phthalates	43,44
Surfactants (e.g.alkylphenol ethoxylates and alkylphenols)	44,45
Synthetic steroids (e.g. DES; ethynyl estradiol)	47
Phytoestrogens (e.g. genistein, coumestrol, etc.)	11-14

There are a wide variety of compounds in the environment that have been shown to bind to the ER and function as either agonists or antagonists. These include both natural products (11-14) and synthetic compounds (3,4). The synthetic compounds include both chlorinated and non-chlorinated compounds (3,4). Some act as xenoestrogens which either mimic or antagonize the effects of endogenous estrogen (Table 1). Others act as androgens in the case of tributyltin (TBT) or as anti-androgens in the case of the fungicide vinclozolin (15) and 2,2-bis(*p*-chlorophenyl)-1,1 dichloroethane (*p, p'*-DDE) (16). Some compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can modulate a number of hormone functions, by acting as both estrogens and anti-androgens (17), as well as affecting thyroid hormone function (18,19) epidermal growth factor (EGF) (10), insulin/insulin-like growth factor-I (20), transforming growth factor- α (21) or other signal transduction pathways including protein kinases (22-26). Thus, it can be seen that a wide variety of types of xenobiotics can exert endocrine modulating effects through many different mechanisms

including altered steroid receptor function, estrogen-androgen ratios, and changes in concentrations of hormones in specific tissues.

Of all the endocrine modulating compounds, those that are direct-acting estrogen receptor (ER) agonists (xenoestrogens or estrogen mimics), direct acting ER antagonists (antiestrogens), or androgen antagonists have received the greatest attention (27,28). This is due to their importance in embryonic development (29). It also reflects the fact that some xenobiotics that have been released into the environment seem to act through mechanisms that affect the expression of sex steroid hormones (30,31). Examples of some compounds that have received attention as potential xenoestrogens or antiestrogens are presented (Table 1). The “estrogen hypothesis” stemmed from the observation that some of the effects observed in oviparous wildlife exposed to persistent and bioaccumulative chemicals were similar to those that could be caused by injecting estrogen into eggs. This hypothesis was supported by the fact that naturally occurring exoestrogens, such as phytoestrogens, could cause reproductive dysfunction in animals (47,48). Further support came from the observation that some xenobiotics which can bind the ER were weak estrogen agonists or antagonists in *in vitro* expression assays (6,27).

Estrogen agonists are compounds that mimic the effects of estrogen. The classic definition of an estrogen is a compound that produces changes in an estrogen-responsive tissue, such as cornification of vaginal epithelium (49). Other physiologic endpoints have been used to define estrogenicity including increased uterine weight, uterine glycogen content, protein induction (50) and cellular proliferation (51). It has been recognized that numerous synthetic as well as naturally-occurring compounds fit the classic definition of an estrogen (52). A refined definition of an estrogen, recognizing the role of nuclear hormone

receptors in gene expression, is a compound that binds to the estrogen receptor, induces dimerization of the receptors that specifically bind to and activate transcription of genes under the regulatory control of trans-acting estrogen responsive elements (53).

Estrogen antagonists block the action of estrogens by interfering with the normal functioning of the estrogen receptor. Anti-estrogenic compounds act by several mechanisms, not necessarily related to estrogen receptor binding and activation. Classical estrogen antagonists such as ICI 164,384 and tamoxifen bind competitively to the estrogen receptor, displacing the natural ligand E₂, and blocking or reducing the effectiveness of the ligand-bound receptor to enhance gene expression (54-56). Aryl hydrocarbon receptor agonists, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and non-*ortho*-chlorinated PCBs, cause down regulation of estrogen receptor and may also interfere with DNA binding (6). Aromatase inhibitors, such as aminoglutethimide, block conversion of testosterone to E₂ and are used in the treatment of metastatic estrogen-dependent breast cancer (57). Inducers of Phase I and Phase II metabolic detoxification enzymes, such as 2,3,7,8-TCDD and non-*ortho*-chlorinated PCBs, reduce the estrogen-dependent expression of proteins by enhancing the enzymatic conversion of latent E₂, thus eliciting an anti-estrogenic response (37).

Another important factor in determining a compound's ability to modulate ER function is bioavailability. Three of the most important characteristics for determining bioavailability of ER ligands are lipid solubility, biological half-life, and amount of protein binding. Comparisons of these properties for natural estrogens, synthetic estrogens, phytoestrogens, and *o,p'*-DDT are presented in Table 2. Some researchers have suggested that xenoestrogens have greater bioavailability than E₂, due mostly to differences in protein

Table 2. *Comparison of factors affecting bioavailability of estrogenic compounds.*

Compound	Lipid Solubility (log K _{ow})	Biological Half-life (h)	Amount of Protein Binding			Amount Unbound
			Steroid Hormone Binding Globulin	Albumin and Other Serum Proteins		
17-β-Estradiol (E ₂)	1.99 (Ref. 58)	13.5 h (Ref. 59)	Men (Ref. 61)	19.6%	78.0 %	2.32 %
			Women (Ref. 61)			
			Follicular	37.3%	60.8 %	1.81 %
			Luteal	37.0%	61.1 %	1.82 %
			3 rd Trimester	87.8%	11.7 %	0.49 %
Diethylstilbestrol (DES)	—	24 h (Ref. 61)	slight (Ref. 60)	10 to 20-fold greater affinity than E ₂ ; high capacity, low affinity binding		—
Genistein		< 12 h (Ref. 62)	27 % relative binding affinity compared to E ₂ (Ref. 63)		—	—
Technical DDT	7.48 (Ref. 64)	—	Unknown	high capacity, low affinity binding ⁶⁵		—

binding and biological half-lives (66,67). It is important to note, however, that this hypothesis has yet to be tested *in vivo*.

It has been suggested that relative to E₂, *o,p'*-DDT, a weak estrogen agonist, may be more active *in vivo* since E₂ binds to steroid hormone binding globulins (SHBGs). Because it does not bind to SHBGs *o,p'*-DDT is thought to be free in plasma and thus more bioavailable. Except in the third trimester of pregnancy, however, less than 40% of E₂ is bound to SHBGs (Table 2). Most E₂ is bound to albumin and other serum proteins, with only about 2% being available as free E₂ (Table 2). While exact values for *o,p'*-DDT distribution in blood are not available, there is a high capacity for binding to serum proteins (48). Furthermore, DDT is more lipophilic than E₂. The octanol-water partitioning coefficient (K_{ow}) for DDT is over 300,000 times that of E₂ (58,61). Thus, DDT is likely to be bound as tightly as E₂, and may, in fact, be less available. Assuming differences on the order of two orders of magnitude are significant for risk assessment, efforts should be made to determine the binding characteristics of xenobiotics relative to E₂.

Description of the ER

The estrogen receptor (ER) is an intracellular receptor belonging to the steroid hormone receptor superfamily (68,69). It functions as a ligand-activated transcription factor mediating the effects of estrogens, which regulate the growth, differentiation, and functioning of diverse target tissues. To date, two isoforms, ER- α and ER- β , have been described (69,70). ER- α has been well-characterized and is a highly conserved protein of approximately 66 kDa (69). ER- β has only recently been described and has a calculated

molecular weight of 54.2 kDa (70). The ligand binding specificities are similar between the two isoforms, however, there are differences in the distribution and relative binding affinities which could contribute to selective actions of ER agonists and antagonists in different tissues (70).

Mechanism of Action For Estrogen Receptor Agonists/Antagonists

A simplified mechanism of action for activation of the ER is shown in figure 1. First, an estrogen receptor ligand must enter the cell and bind to the ER. The non-ligand bound form of the ER is predominantly localized to the nucleus (71) and is part of a complex with associated proteins (72). Upon ligand binding, the associated proteins dissociate, and then ligand-receptor complexes associate with additional nuclear factors (73) and bind to estrogen responsive elements (EREs) as homodimers. Binding of the transformed complex to the ERE results in production of mRNA for a number of estrogen-responsive proteins, such as PS-2, and cathepsin D (6).

In addition to endogenous responses, exogenous reporter systems, such as firefly luciferase under control of EREs, can be stably transfected into cells such that upon exposure to estrogens, luciferase expression is induced (74). Luciferase or other such reporter systems provide a rapid and convenient means for receptor-mediated dose-response assessment as well as allowing for mechanistic investigations into transcriptional activation.

There is considerable evidence for hyperphosphorylation of the ER after ligand binding, supporting the hypothesis that phosphorylation is an important regulatory mechanism for ER function. Phosphorylation of key serines (75-77) and at least one

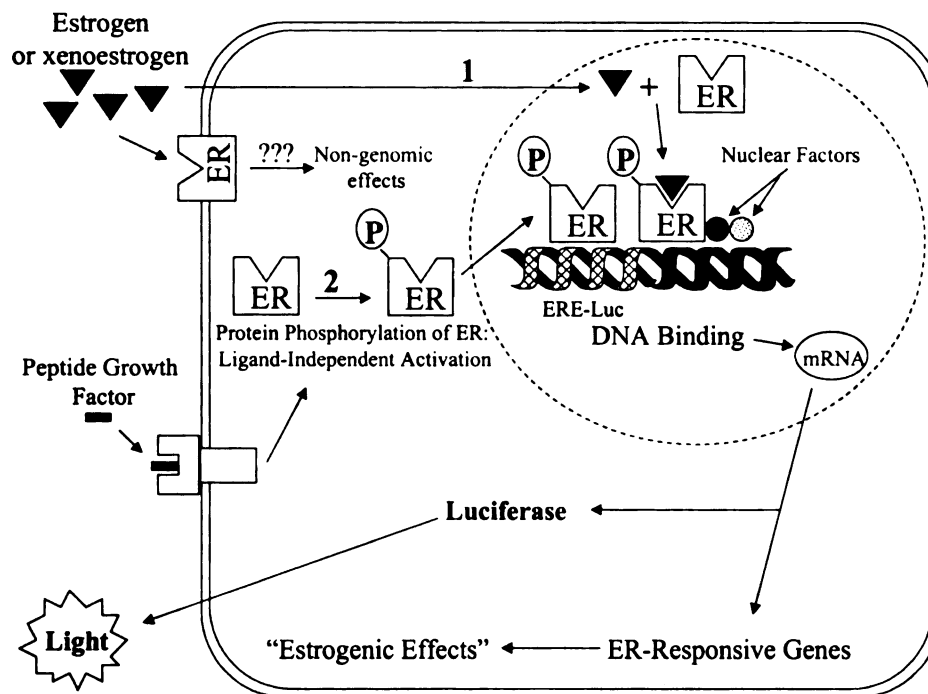


Fig. 1. Model depicting mechanisms for activation of the estrogen receptor (ER) in an MCF-7 cell line stably transfected with an ER-controlled luciferase reporter gene construct (MCF-7-luc). Refer to the text for a full description. In brief, pathway 1 depicts an ER agonist entering a cell and interacting with intracellular ER, which then binds to estrogen responsive elements (EREs) in the promoter region of ER-responsive genes. Pathway 2 depicts ligand-independent activation of the ER through a protein phosphorylation pathway.

tyrosine residue of the ER, Tyr 527 (78), play an important role in regulating the transcriptional activity of the ER. Phosphorylation increases the negative charge and acidity of a region of a protein, thereby modifying interactions with other proteins and DNA. Hypo- and hyper-phosphorylation at the same time in different regions of the ER could potentially explain differential transcriptional regulation of certain genes, in addition to tissue- and cell-specific regulatory mechanisms (79). In addition, differential phosphorylation of other nuclear factors with which the ER interacts to mediate transcription potentially play a significant role (80).

There are also ligand-independent pathways for modulating the transcriptional activity of the ER through activation of protein kinases (Fig. 1, pathway #2). For example, upon treatment with growth factors, such as epidermal growth factor, insulin-like growth factor-1, and platelet-derived growth factor, or agents that increase cAMP levels, the ER can be phosphorylated, bind to EREs, and activate transcription in the *absence* of ligand (81-83). The fact that the pure antiestrogen ICI 164,384 blocks the effects of these agents supports an ER-mediated mechanism (83). Likewise, protein kinase inhibitors block the effects of these agents and E₂ (69). ICI 164,384 also stimulates phosphorylation of the ER without a similar increase in transcriptional activation. This indicates that overall phosphorylation does not necessarily result in increased transcriptional activity (69) although this could be due to impaired receptor dimerization by ICI 164,384 (55). Given the fact that some xenobiotics can modulate protein phosphorylation (26,84,85), this ligand-independent mechanism for modulating ER function has a potentially important impact on screening for chemicals with estrogenic activity which will be discussed later, especially if the emphasis is placed on receptor binding alone as a first tier screen. For example, a

TABLE 3. *Relative binding affinity (RBA) for calf estrogen receptor and luciferase expression potency and efficacy in MCF-7-luc cells*

Compound	RBA IC ₅₀ (nM)	MCF-7-luc	
		Potency (ED ₅₀ nM)	Efficacy (fold induction relative to solvent controls)
17β-Estradiol	6.9	0.00625	4.5
Coumestrol	24	2315	2.8
17β-Ethynyl-estradiol	37.3	0.0555	4.5
Nonyl-phenol	2300	8.56	1.9
Octyl-phenol	12300	16	2.3
Bisphenol A	24300	3640	4.3
Indole-3-Carbinol	2.3x10 ¹⁰	21100	2.3
Atrazine	>2.3x10 ¹⁰	2470	2.8
o,p'-DDE	>2.3x10 ¹⁰	NE	—
p,p'-DDE	>2.3x10 ¹⁰	NE	—
2,3,7,8-TCDD	>2.3x10 ¹⁰	NE	—

IC₅₀-concentration of competitor to reduce total binding control counts by 50%; ED₅₀-dose to elicit 50% of maximal effect. NE, potency estimate was not possible based on the dose-response obtained.

compound like 2,3,7,8-tetrachlorodibenzo-p-dioxin, which is a potent antiestrogen (86), would be missed in an initial screen because it does not bind to the ER (87). Similarly, the triazine herbicide atrazine, which does not bind to the ER, can cause estrogen-like responses in *in vitro* expression assays (Table 3). Neither of these *in vivo* or *in vitro* effects would be predicted from receptor binding assays.

Rapid, Non-genomic Effects of Estrogens

In addition to the mechanisms described above, estrogens produce rapid (within seconds to minutes), non-transcriptional responses which are similar to those evoked by growth factors. Recent evidence suggests the existence of a membrane ER (88) which may play a role in these rapid, non-genomic effects of estrogens, which include prolactin release in GH3/B6 rat pituitary tumor cells (88), intracellular calcium release in chicken granuloma cells (89), stimulation of protein tyrosine phosphorylation in MCF-7 mammary carcinoma cells (90) activation of the p21^{ras}/MAP kinase pathway in MCF-7 cells (91) and stimulation of adenylate cyclase and cAMP-regulated gene transcription (75).

Interactions and Cross-talk from other Signaling Pathways

“Cross-talk” between the estrogen receptor and other signaling pathways provide important mechanisms for modulating biological responses. Interactions and “cross-talk” with the ER have been described for the progesterone receptor (69), aryl hydrocarbon (Ah) receptor (92), epidermal growth factor receptor (81), insulin-like growth factor I (69), and as discussed above, pathways involving agents affecting protein kinase

activities, particularly those affecting protein kinase C (PKC), cAMP-dependent protein kinase (PKA), and tyrosine kinases (81,83).

As interest in monitoring the environment for environmental estrogens increases, it is likely that more than one mechanism will need to be assessed. Some environmental mixtures contain compounds that modulate the responsiveness of multiple receptor-mediated pathways, such as the aryl hydrocarbon (Ah) receptor, estrogen receptor, androgen receptor, epidermal growth factor receptor, *etc.* Thus, the complexities of environmental mixtures require innovative methods and approaches to assess the potential for adverse effects.

SCREENING AND MONITORING

Concern over xenoestrogens has created a need to both screen and monitor for compounds which can modulate endocrine effects. This need is underscored by recent legislation mandating that chemicals and formulations be screened *a priori* for potential estrogenic activity before they are manufactured or used in certain processes (Safe Drinking Water Act Amendments of 1995 - Bill Number S.1316; Food Quality Protection Act of 1996 - Bill Number P.L. 104-170). Monitoring *a posteriori* is needed in order to assess concentrations of estrogenic compounds in both abiotic matrices like soil, sediments, and water, and biotic matrices such as human and wildlife tissues and food stuffs (93,94).

Both biological and instrumental methods can be applied to monitor or screen for estrogenic compounds. Instrumental methods are useful for measuring the uptake, disposition, and concentrations of specific compounds (monitoring). They can also be applied to help discern metabolic pathways. They are generally not useful, however, for discerning biological efficacy (screening). A variety of bioassays, both *in vitro* and *in vivo*

can be used to screen individual compounds, formulations, or environmental samples for potential estrogenic/antiestrogenic activity. Models may also be used to predict the potential estrogenicity based on the physico/chemical properties of the compound of interest.

A number of techniques for screening and monitoring for the effects of xenoestrogens are discussed below, with most attention being focused on bioassay methods. The advantages and disadvantages of each technique are discussed. Although this discussion is focused on assays used to determine the estrogenicity or anti-estrogenicity of a given compound or sample, the same type of techniques can be applied to other receptor-mediated processes, as long as the mechanism of action is known.

Predictive Quantitative Structure Activity Relationships

The ideal screening tool is one that is rapid, inexpensive, objective, applicable for a wide range of compounds acting through a given mechanism of action, and capable of accurately predicting a compound's potential to elicit (adverse) effects *in vivo*. One of the most attractive screening tools is computer modeling, based on quantitative structure activity relationships (QSAR). Once a suitable computer model is constructed, compounds can potentially be screened for possible activity in less time than it takes to conduct even the most simple *in vitro* bioassays, and at a fraction of the cost. For this reason, development of an accurate QSAR model is certain to be a goal of any large-scale screening program. Some of the disadvantages of such models is the difficulty in collecting necessary input parameters that are representative of all potential ER ligands, and the

reliance on receptor binding assays in many of these models rather than looking at ER function (to be discussed in more detail in following sections).

Structure activity relationships for estrogenic compounds have been studied extensively (50,51,95,96). Correlations have been made between certain structural features and both affinity for the estrogen receptor, and expression of estrogen modulated genes (50,95,96). Evidence of stereochemical recognition and stereospecific modulation of gene expression has been reported (50). Though estrogenic substances vary widely in structure, some common characteristics of most estrogens include 1) a sterically unhindered phenol group, and; 2) a hydrophobic substituent of greater than three carbons bonded *para* to the phenolic hydroxyl (56,97). The aromatic A-ring with its free hydroxyl group has been cited as a key for affinity of endogenous steroid hormones to the estrogen receptor (95,96). Structure activity relationships identified have been based on both simple structural features, and more advanced computer based models such as the electrostatic models used by VanderKuur *et al.* (96); comparative molecular field analysis (CoMFA) used by Waller *et al.* (98,99); and computer graphic and energy based models for fit into DNA used by Hendry *et al.* (100,101).

QSAR-based computer models for predicting estrogenic and/or androgenic potential have been developed. Two of the most promising models are discussed here. CoMFA/3D-QSAR based models for predicting ER or AR binding affinity have been proposed (98,99). These models consider the overall steric and electrostatic properties of the compound of interest. Empirically derived ER binding affinity for seven classes of potentially estrogenic compounds, both natural and synthetic were used for the construction and validation of the model for ER binding affinity (98). Average errors of less than 2 log

units for predicting empirically derived ER or AR affinity based on the CoMFA/3D-QSAR model have been reported (98,99). Such a range of error may or may not be within the range of certainty required by a tier I screening tool.

Another QSAR approach has been proposed by Hendry *et al.* (100,101). This approach is based on the hypothesis that ligand fit into DNA (or DNA complementarity) is an important property affecting hormone-like activity *in vivo* (100,101). The model developed uses both 3D computer graphics and energy (electrostatic and van der Waals) calculations to estimate fit into DNA (100,101). Degree of fit into DNA has been found to correlate with *in vivo* responses to estrogens such as uterotrophic activity (101) and *in vitro* responses like proliferation of MCF-7 human breast cancer cells (100). Hendry *et al.* found that, without exception, compounds which fit into DNA better than E₂ were more active *in vivo* than E₂, while those that fit more poorly exhibited less uterotrophic activity *in vivo* (101).

Both of the models discussed above appear to hold promise as potential tools for screening compounds for potential estrogenic or endocrine modulating activity. QSAR approaches could be used in a tiered screening system for direct-acting estrogenic compounds acting as agonists or antagonists. QSAR models based on receptor binding affinity alone, will have little or no utility in identifying estrogenic compounds acting through non-ER mediated mechanisms. Furthermore, additional validation will be necessary before QSAR techniques can be applied with certainty. Both models need to be validated with a larger set of compounds. Additional *in vitro* and *in vivo* studies are needed to establish relationships between predicted receptor or DNA fit and relevant *in vivo* endpoints in multiple species. Finally, if they are to be applied, such models should be used

as a tier I tool to narrow the range of compounds to be examined more thoroughly using *in vitro* and *in vivo* bioassays. Regardless of their utility as screening tools, QSAR approaches are useful in understanding of mechanisms and scaling doses in experiments.

***In Vitro* Assays**

There are a number of types of *in vitro* assays available for measuring the estrogenic activity of single compounds or complex mixtures (102). The range of responses includes everything from simple receptor binding to expression of endogenous or exogenous genes to cell proliferation and differentiation (52). *In vitro* systems are attractive as screening tools because they are rapid, inexpensive, and fairly reproducible. For these reasons precise estimates of the relative potency of a great many samples or compounds can be obtained in a rather short period of time. One of the greatest utilities of *in vitro* assays is for studying mechanisms of action of compounds or assessing the potential for synergisms among direct-acting estrogen agonists. Using simple *in vitro* cell systems it is often possible to determine the mechanism of action of a compound. Knowledge of a compounds mechanism of action can reveal ways to monitor using *in vivo* biomarkers.

In vitro assays are limited by the fact that they do not completely represent the *in vivo* situation. Pharmacokinetics, biotransformation, and binding to carrier proteins may not be accurately represented by *in vitro* systems. In addition, some xenoestrogens may be activated or deactivated by enzymatic conversion during metabolism, conjugation, and/or excretion. While there are methods to adjust for and minimize some of these limitations, they must be considered when applying the results of *in vitro* screening tests.

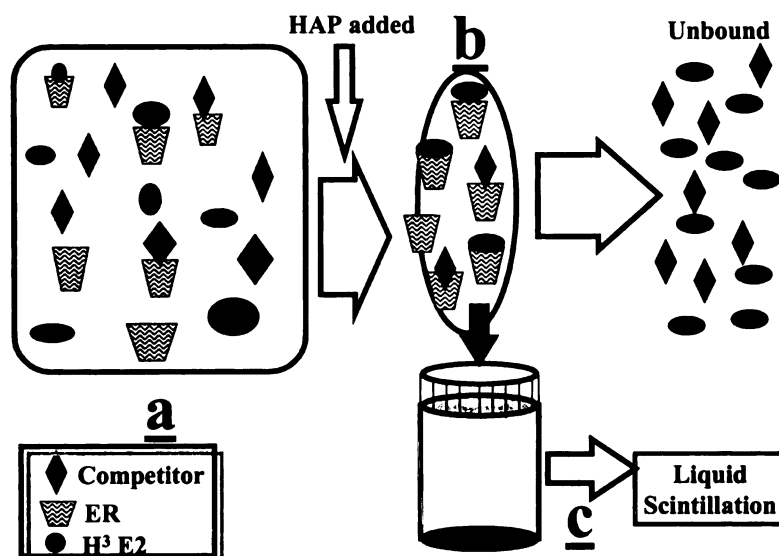


Fig. 2. Competitive receptor binding assay. a) Competitor (compound being tested) competes with tritiated 17- β -estradiol ($^3\text{H}^3\text{E}_2$) for binding to calf uterine estrogen receptor (ER) in a test tube. b) Hydroxyapatite (HAP) is added to complex with proteins (and bound ligands). Proteins with their bound ligands are trapped on filter paper while the unbound ligand passes through. c) Filter paper transferred to a scintillation vial and counted. Successful competitors cause reduction in counts relative to a control using $^3\text{H}^3\text{E}_2$ only.

Receptor Binding Assays

For direct-acting estrogen agonists or antagonists to exert an effect, it is necessary for them to bind to the estrogen receptor (103). The affinity with which such compounds bind to the estrogen receptor may be related to the potency of the compound relative to endogenous estrogen. For this reason, receptor binding assays have been used as a method to screen for potential estrogenic compounds (16,44). It is important to note, however, that while binding of the ligand to the ER is necessary, it alone may not be sufficient for eliciting estrogenic responses in tissues.

Receptor binding assays are conducted as competitive binding assays where a compound with unknown binding affinity is allowed to compete for estrogen receptor binding sites with a labeled standard of known affinity (Fig. 2). Operationally, a known amount of the compound of interest is added at varying concentrations to a fixed amount of receptor and competitor. The compound of interest is allowed to compete with radiolabelled competitor for a fixed number of binding sites. (Fig 2 - a). The receptor-ligand complexes are separated from suspension by filtration (Fig. 2 - b) or centrifugation and the amount of activity determined via liquid scintillation (Fig. 2 - c) (104). Compounds with affinity for the estrogen receptor will “displace” or compete for more of the available binding sites, thereby decreasing the radioactivity of the receptor-ligand complex fraction accordingly. Scatchard (105) and/or Woolf (106) analyses can be used to determine the concentration of compound required to displace 50% of the endogenous compound (hormone) or its synthetic analogue. Relative binding affinities can be derived for compounds of interest. These values, generally reported as the effective concentration needed to reduce the binding of the labeled competitor by 50% (IC_{50}), are measures of the relative potency of the

compound. For instance, if the concentration of a compound required to compete for 50% of the binding sites is 1000 times (on a molar basis) greater than the endogenous or synthetic reference compound, it would have a relative potency 1000 fold less than the reference compound. In theory, this information can be used to predict concentrations of competing compounds in organisms that would be required to cause a given level of effect. Several competitors have been used in receptor binding assays. These include endogenous ligands such as, E₂ (14,63), in the case of the ER. Alternatively, synthetic competitors such as ethinylestradiol (EE₂) or diethylstilbestrol (DES) can be used. The use of synthetic competitors is useful because they are often more stable and are not as likely to bind non-specifically to endogenous non-receptor proteins.

Receptor binding assays are attractive because they are simple and inexpensive to conduct. The only materials necessary are a preparation of the purified receptor of interest and an agonist of known binding affinity. In the case of the estrogen receptor (ER) the receptor is similar among species (107). Thus, results obtained with a preparation from one vertebrate species can generally be extrapolated to other species. However, this may not be true for all receptor-mediated responses. ER preparations from estrogen-responsive tissues of rodents, calf, humans and other vertebrates such as fish have been used in binding assays (44,47,108). In addition, receptor does not necessarily need to be harvested from animals or their tissues. Receptor can also be harvested from *in vitro* cell cultures. Transformed yeast cells (109,110) and transfected cell lines (14) have been used to produce relatively large quantities of uniform receptor.

Although they are attractive and simple, receptor binding assays are limited in the amount of information they can provide. The affinity of binding to a receptor is only one

step in a complex series of events that occur during endocrine modulation (Fig. 1). In addition, there needs to be transformation and translocation of the receptor followed by binding to accessory ligands or proteins and subsequent transcription (Fig. 1). Receptor binding assays cannot account for such steps in the expression process. In addition, a compound may be present in sufficiently great concentrations to cause effects even though it has a relatively weak affinity for the receptor. *In vitro* receptor binding assays do not consider pharmacokinetic processes that are important determinants of exposure *in vivo*. Enzymatic modification, differential turnover rates, and binding of endogenous and exogenous ligands are often different in the *in vitro* and *in vivo* situation. Finally, from binding affinity one can not infer whether a compound will be an agonist or antagonist. One can only identify the potential for modulation of the receptor-mediated process.

Relationships Between In Vitro Receptor Binding and In Vivo Assays

For simple *in vitro* bioassays such as receptor binding to be useful for screening, it is necessary for the responses to be correlated to physiologically-relevant responses *in vivo*. Risk assessment based on receptor binding affinity would be meaningless without such correlation. Thus, the use of receptor binding affinity as a predictive tool is predicated on the assumption that endocrine modulation proceeds through a receptor-mediated process (111). There may, however, be alternative signal transduction pathways. Here, the relationship between receptor binding affinity and *in vitro* gene expression will be discussed.

In an attempt to elucidate the potential for receptor binding assays to predict effects *in vivo*, affinity for binding to calf uterine ER was compared to responses in a simple *in vitro* gene expression assay (discussed in more detail later in this chapter). The expression

assay used MCF-7-luc cells which are MCF-7 human breast tumor cells (ATCC # HTB-22) stably transfected with a DNA construct that includes an exogenous reporter gene, luciferase, under control of estrogen responsive elements (EREs) and a mouse mammary tumor virus (mmtv) promoter (74). The comparison was made for a range of potentially estrogenic compounds including endogenous estrogen, synthetic estrogen, xenoestrogens and a natural product (Table 3). The hypothesis that receptor binding affinity can be used to predict ERE mediated gene expression *in vitro* was tested, to gain insight regarding the potential predictive power of receptor binding assays. Results indicated that relative binding affinity (RBA) for calf uterine ER was correlated with the *potency* for expression in the MCF-7-luc bioassay ($r^2=0.711$; Table 3). RBA was not, however, very predictive of *efficacy*, or magnitude of expression, ($r^2= -0.356$). Furthermore, there was no relationship between potency and efficacy among the compounds studied using the expression assay ($r^2= -0.165$). Since the response of an organism is a complex interaction between both potency and efficacy, these results suggest that screening of compounds or mixtures with a simple receptor binding assay will not be very predictive of other *in vitro* or *in vivo* responses.

Results from this *in vitro* study correspond with reports in the literature that suggest a lack of correlation between RBA and ligand activity *in vivo* (112,113). It has been stated that “interaction with the estrogen receptor may not necessarily be an absolute, or sole requirement for the expression of estrogenic activity” (114). It has also been reported that there is little relationship between receptor binding of E₂ analogues and either the character or extent of response (95). Similarly, a poor relationship between receptor binding and biological activity of non-steroidal DES analogues has been found. Researchers have concluded that ligand structure may be important in processes other than binding to and

activating the ER (50). Alkaline degradation products of some steroids are potent estrogens, even though they do not bind with great affinity to the ER (115). One of the most active estrogen analogues developed has a receptor binding affinity of less than 1% of that of E₂ (116). The triazine herbicide atrazine was found to have no measurable affinity for the ER, yet caused a significant response in the MCF-7-luc assay (Table 3). Ligand independent activation of the ER by growth factor signaling and protein kinase activation can occur (69). Some potent antiestrogens bind the ER poorly or not at all (117,118). In some cases there is a need for metabolic activation of compounds, thus receptor binding affinity of the parent compound may not relate to *in vivo* activity (112,119).

In vitro and *in vivo* estrogenicity of *o,p'*-DDT, *o,p'*-DDE and chlordecone, based on vitellogenesis in fish, has been compared (33). While both *in vitro* (ER binding affinity) and *in vivo* (plasma vitellogenin) assays indicated that these compounds were weakly estrogenic, the potency of the substances, based on the two assays was not correlated. ER binding studies indicated that *o,p'*-DDT and *o,p'*-DDE were less potent competitors for *moxestrol* (synthetic estrogen) than was chlordecone. However, *in vivo* studies, based on plasma vitellogenin normalized for tissue residue levels, indicated that *o,p'*-DDT and *o,p'*-DDE were more potent than chlordecone.

Together, the results of our studies and reports in the literature suggest that the assumption upon which application of receptor binding assays is based may not be valid. The information available suggests that ER binding may be a poor predictor of more complex *in vitro* and *in vivo* responses. One reason for the discrepancy between receptor binding and hormonal activity *in vitro* could be the multiple steps involved in expression beyond receptor binding. These include both transcriptional and post-transcriptional events.

Lack of concordance between receptor binding assays and *in vivo* responses may also be due to nongenomic mechanisms or influences through other pathways, which were discussed earlier in this chapter. Slight differences in receptors or receptor expression among different species and tissues (79,120,121) must also be considered a potential source of the discrepancy. While the structure of the ER is well conserved among species, there are species, tissue-specific, and even temporal differences in the affinity to estrogen agonists (69,121).

One alternative working hypothesis that could explain the lack of correlation between binding affinity and response is the “ligand insertion hypothesis” (122). This hypothesis states that upon binding to both the ligand and the DNA (at the ERE), the receptor shifts conformation in a way that facilitates insertion and release of the ligand into the DNA where it acts as an additional transcription factor (122). Thus, the characteristic structure of the compound and its fit into DNA would influence transcriptional activity in a manner not necessarily related to its receptor binding affinity (122). The results reported here do not provide a rigorous test of this hypothesis, but they are consistent with it. Binding affinity could determine the concentration of ligand needed to produce a response (potency) while the structure of the ligand itself would determine its effectiveness as a transcription factor (which would have bearing on the magnitude of response generated - efficacy). This hypothesis is only now being developed and tested with computer modeling and simple expression assays. If it is validated it would further support the contention that measures of receptor binding would be poor predictors of the effects of endocrine disrupters. In light of the information reviewed here, the use of empirically determined receptor binding affinity is unlikely to be very accurate for screening

and risk assessment purposes. Furthermore, QSAR relationships based solely on ER binding may not be sufficiently accurate to be used in a tiered screening approach.

Cell proliferation and differentiation

One of the *in vitro* assays most frequently used to determine the relative potency for EDCs acting through the ER is cell proliferation of estrogen responsive tissues (123). The MCF-7 and ZR-75 cell lines are human breast cancer cells that have been used extensively for screening of estrogenic effects (34,44,124). The primary cell line used to assess estrogenic effects is the MCF-7 line, which was originally derived from a hormone-dependent metastatic breast cancer (125). These cells were demonstrated to contain ER (126) and express a number of responses that are under control of the ER. MCF-7 cells have been used in the *E-screen* to screen for estrogenicity of a number of compounds (34,51,127,128). This assay is based on estrogen (E_2)-dependent proliferation of MCF-7 cells.

In the *E-screen* assay, MCF-7 cells are cultured in media stripped with dextran coated charcoal so that it is E_2 -deficient. A test compound is interpreted as an estrogen agonist if it significantly increases cell proliferation relative to a control upon its addition to the stripped media. In the original *E-screen* assay the response was determined by counting cell nuclei in trypsinized cells (129). Other responses of cell proliferation such as metabolic reduction of dimethylthiazolyldiphenyltriazolium bromide (MMT), reduction of thiamine blue, and incorporation of [^3H]thymidine have also been used.

The *E-screen* assay has been extensively used (15,34,42,124), but it has some limitations. Since it measures cell proliferation, the *E-screen* assay is, technically, a

mitogenicity assay. Thus, a positive response can not be strictly attributed to estrogen agonists. Variation in media preparation and relative concentrations of E₂ and other growth factors can greatly affect the responses obtained by different laboratories. Also, because it depends on a proliferation response, ER antagonists are not easily detected using this assay. Furthermore, secondary types of endocrine modulators that could result in responses that are similar to those involving E₂ at the ER (such as anti-androgens) would not be measured by this assay. For these reasons, if used alone, the *E-screen* assay could result in a significant number of false negative determinations of EDCs. A positive response in the *E-screen* assay should be confirmed by *in vivo* studies before a conclusion about environmental activity of a compound is made.

Expression Assays

Because it has been demonstrated that, in some situations, binding of a ligand to the ER is necessary but not sufficient for endocrine modulation while in other situations modulation can occur without significant ER binding, more complex *in vitro* assays have been developed. Knowledge of ER binding characteristics is only part of the information necessary to interpret the potential endocrine modulatory effects of compounds. A compound that binds with high affinity may be either an agonist or an antagonist. Furthermore, proliferation is a potentially nonspecific response. For this reason a series of *in vitro* receptor-dependent transcriptional expression assays have been developed. Those designed to measure potency of estrogenic, antiestrogenic, androgenic, and anti-androgenic compounds are the best developed. A number of these types of systems have been developed for ER-dependent responses.

MCF-7-luc Bioassay Method

Expression assays examine induction or suppression of proteins encoded by genes whose transcription is thought to be modulated through an ER mediated mechanism. Increases or decreases in the activity of the protein of interest upon exposure to a single compound or complex mixture, such as an environmental extract, suggest the presence of one or more ligands with the potential to modulate a broad range of genomically controlled estrogenic responses. The MCF-7-luc bioassay method (see discussion of receptor binding, this chapter) is detailed here as a prototypic expression assay (Fig. 1). MCF-7-luc cells are seeded into 96 well microplates. Compounds or environmental samples are then delivered to the plates. Active compounds enter the cell where they affect the transcription of ERE regulated genes, presumably through mechanisms discussed earlier in this chapter. Estrogen agonists cause upregulation of transcription of the luciferase reporter gene. Since there is sufficient E₂ in the medium to allow constitutive luciferase expression, the assay can also be used to detect antagonists (103). Luciferase mRNA is produced and translated at the ribosomes into the luciferase enzyme. The activity of this enzyme is then measured by adding the exogenous substrate luciferin which interacts with luciferase in a light producing reaction. The magnitude of light production, measured by a luminometer, serves as a gauge of reporter gene expression and thus a gauge of the estrogenic potential of the sample. Potency of the sample can be reported in terms of the concentration of test substance needed to yield a significant amount of light (LOEC) or concentration needed to yield 50% of maximal light production (EC-50). The maximum amount of light produced provides a measure of the compound's efficacy. This means that relative potencies of both agonists and

antagonists can be determined singly or as a net potency for a complex mixture of both agonists and antagonists. Though there are other expression assays (6), the MCF-7-luc method illustrates many of the features common to expression assays (*i.e.* examines changes in expression of a gene whose activity is modulated by a known mechanism of action upon exposure to a ligand). Most of the differences among expression assays are the result of differences in the particular cells and/or reporter gene/protein, used in the assay system.

Applications of the MCF-7-luc Bioassay (a Model Expression Assay)

The MCF-7-luc bioassay has been successfully utilized to screen for both estrogen agonists and antagonists, assess interactions within mixtures, conduct mechanistic studies, and perform environmental monitoring of a wide variety of biotic and abiotic matrices. Recently, there has been controversy over the potential for mixtures of weakly estrogenic pesticides to act in a synergistic (130) or additive manner (131). When tested in the MCF-7-luc, dieldrin, endosulfan I, and a 1:1 mixture of endosulfan I and dieldrin (E+D) all displayed similar dose-response curves (Fig. 3). Potencies for individual compounds and the binary mixture were not significantly different and were approximately a million-fold less potent than E₂. Thus, synergism was not observed between endosulfan I and dieldrin in MCF-7-luc cells. In addition, it is important to point out that the magnitude of response (efficacy) elicited by these

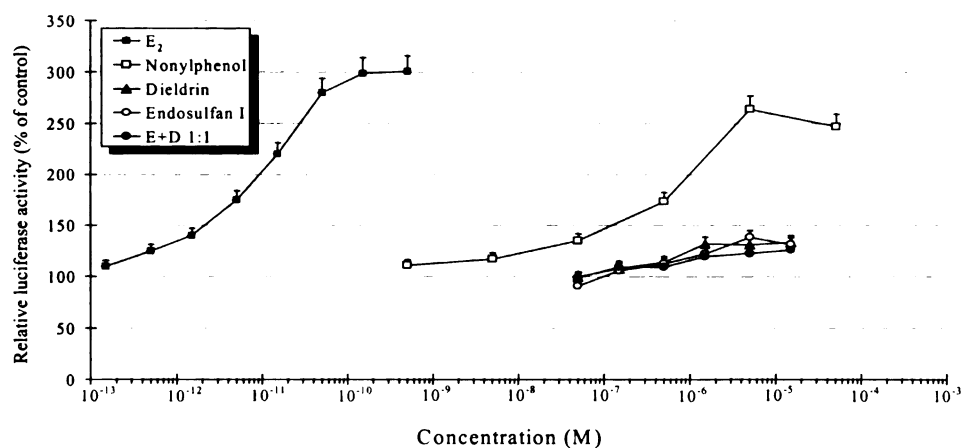


Fig. 3. Estrogen-responsive element-mediated induction of luciferase activity in MCF-7-luc cells. Cells were treated with 17- β -estradiol (E₂), nonylphenol, dieldrin, endosulfan I, dieldrin plus endosulfan I (E+D 1:1 mixture), or solvent only for 3 d. Relative luciferase activity is expressed as a percentage of control, with each point representing the mean of at least three replicates (standard deviations are represented by error bars).

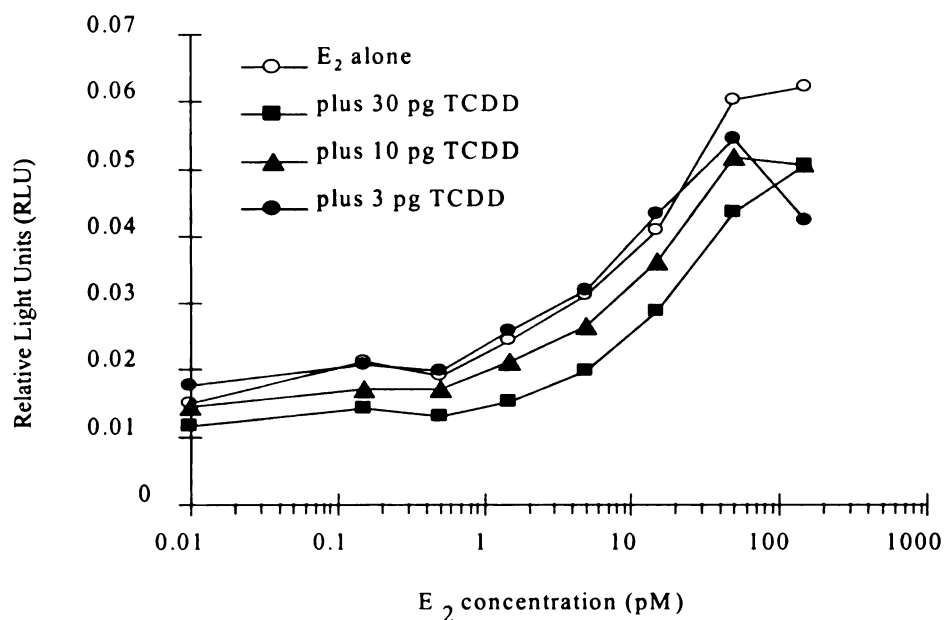


Fig. 4. Dose-dependent inhibition of estrogen-responsive element-mediated induction of luciferase activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7-luc cells. Cells were treated with E₂ either alone or in combination with TCDD for 3 d. Relative luciferase activity is expressed as relative light units, with each point representing the mean of at least three replicates (standard deviations are represented by error bars).

chlorinated pesticides is considerably less than that caused by E₂. Another environmental estrogen, nonylphenol, was found to possess an efficacy approximately 90% of E₂ but with a relative potency approximately 10,000-fold less than E₂. Therefore, when comparing estrogenic activities of potential xenoestrogens, it is important to consider both potency *and* efficacy.

Since estrogen antagonist potency can be determined using the MCF-7-luc bioassay, the dose-dependence for TCDD's antiestrogenicity was evaluated for the full dose-response curve of E₂ (Fig. 4). In general, parallel dose-response curves were obtained and there was a dose-dependent increase in the EC₅₀ for E₂ (*i.e.*, a decrease in potency) with increasing concentrations of TCDD.

Other Expression Assays

Cell lines used for expression assays include chicken fibroblasts, both primary and transformed rainbow trout hepatocytes (6,44,132,133), MCF-7 (42,131), HeLA (6,14), yeast (6,130,131), and others. There is considerable evidence that *in vitro* expression of ER modulated genes is influenced by cell and promoter context (69,121,134,135). The type of cell used in the expression assay system largely determines the number of confounding factors that must be considered when interpreting results and determining the relevance of the assay for predicting effects *in vivo*. This is due to differences in the intracellular "machinery" found in different types of cells. Some cells, such as yeast, contain none of the receptors, DNA response elements, or steroid responsive genes involved in the gene expression assay. These cells simply act as housing for exogenous expression systems (6). Others contain parts of the machinery but lack one or more components, such as receptors or

regulatory sequences of DNA. Finally, some cells contain all the machinery necessary for expression.

Yeast, such as *Sacchromyces cerevisiae*, represent the stripped-down type of cell. While they contain no steroid receptors of their own, steroid receptors expressed in yeast have been shown to function normally (136,137). Thus, they must be transformed with plasmids coding for estrogen receptor and a reporter gene to be used for expression assays. Yeast based expression assays have been used by a number of researchers (6,130,136,138-140). They have a number of advantages. First, they tend to be relatively simple and rapid assays since yeast is more easily cultured than more complex cells. Because they have no endogenous hormone responsive machinery, they are specific for compounds that act directly through estrogen receptor mediated control of gene expression. Responses are not modulated by other regulatory factors. This also means that yeast cells are isolated from many of the confounding factors that affect more complex cells (140). The tradeoff, however, is the inability of yeast-based expression systems to account for many of the factors at the cellular level that may affect a compounds activity *in vivo*. Furthermore, yeast cells have some morphological (*i.e.* cell walls) and physiological properties not found in most animal cells (6). These factors may affect the accumulation and response dynamics of the xenobiotics. Indeed, significant differences in response between yeast cell based assays and mammalian cell assays have been reported (6).

Another level of complexity are expression assays which use complex cells such as mammalian hepatoma cells which do not express steroid receptors of their own. Such cells transfected with plasmids coding for estrogen receptor and an appropriate reporter gene retain the specificity of yeast based-assays but incorporate some of the more complex

pharmacokinetics, metabolic potential, etc. of more complicated cells. Such cells are considered particularly useful for studying receptor function (140).

Cells containing all the necessary machinery such as MCF-7 cells or rainbow trout hepatocytes are the most realistic in terms of cellular level factors that can influence expression. This, however, increases the complexity of interpretation by expanding the number of possible avenues via which compounds in a sample may influence the endpoint measured. The choice of whether to use an exogenous or endogenous reporter gene/protein as an endpoint provides another level of complexity/realism within cells containing a complete set of endogenous estrogen modulated gene expression machinery.

The different reporter proteins used as endpoints for expression assays are nearly as numerous as the cell types used. Common reporters include endogenous proteins such as pS2 (42,141) vitellogenin (44,132,133) and sex hormone binding globulins (6) and exogenous reporters like luciferase (74), and β -galactosidase (130,140), and chloramphenicol acetyltransferase (CAT) (14). Like cell type, the type of reporter protein used as an endpoint can affect the number of confounding factors to consider. Endogenous proteins may have a direct affinity for the ligands being studied, may be linked to metabolic processes or feedback mechanisms not directly controlled by the estrogen expression mechanism, may be affected by other steroid hormones, etc. Exogenous proteins like luciferase are less likely to be affected by such factors.

The type of reporter protein or endpoint used also has a major impact on the sensitivity and responsiveness of the assay, since it affects the type of instruments or analytical methods that can be used to detect it. Due to the availability of sensitive detectors for light and the high quantum efficiency of the luciferase reaction, the light producing

endpoint for a luciferase based expression assay can be very sensitively detected using a luminometer (142). The sensitivity of MCF-7 assays using a luminescent endpoint are in the low pM ranges (6). Colorimetric endpoints, such as the β -galactosidase endpoint used in many yeast based assays tend to be nearly 100 fold less sensitive (140).

Expression assays have several advantages over other potential *in vitro* screening assays. First, they tend to be more sensitive than receptor binding assays (Fig. 5) (6), though their degree of sensitivity varies considerably with both cell type and reporter protein used. Unlike receptor binding or cell proliferation assays, expression assays can be used to detect both estrogen agonists and antagonists (14,74,143). Because protein expression requires the transcription of a gene to mRNA followed by translation at the ribosomes, expression assays incorporate transcriptional and post-transcriptional processes that may be important determinants of ligand-influenced response. This means expression assays can detect ligands that influence any part of the gene expression pathway, not just those that bind to the estrogen receptor. This makes such assays more robust in light of uncertainties that remain regarding mechanisms of action. Expression assays should also be more specific than proliferation assays, since many factors that can affect proliferation (*i.e.* temp, pH, serum quality, other mitogens, etc.) should have little or no bearing on expression of estrogen modulated genes.

The primary disadvantages of expression assays are those common to all *in vitro* bioassays. Like all *in vitro* systems, expression assays do not incorporate the entire range of metabolic and pharmacokinetic factors that can affect the disposition of target compounds in a whole organism. The inability to account for such complexities makes the *in vivo*

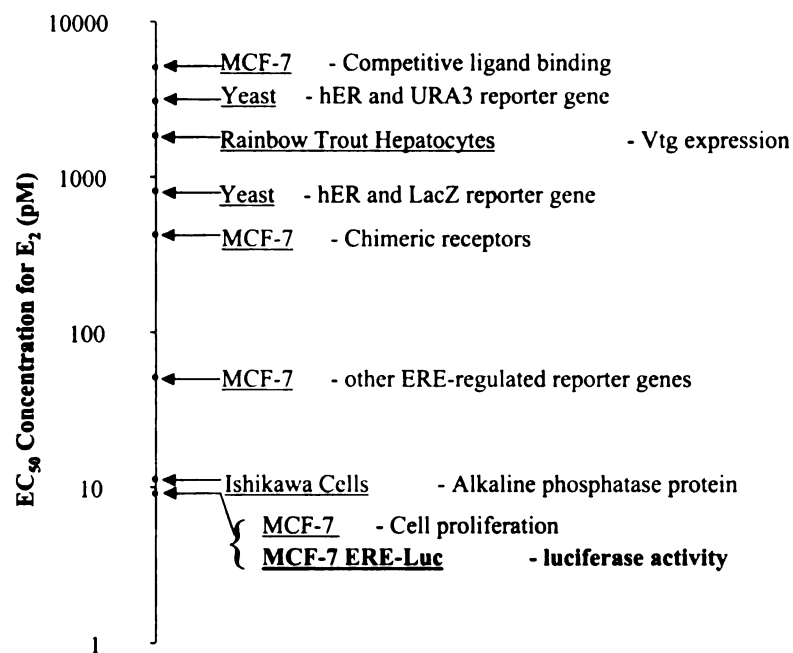


Fig. 5. A comparison of several *in vitro* bioassays for estrogenic activities. Adapted from Zacharewski (6).

relevance of such tests unclear. More work is needed to examine and develop correlations between expression assay results and effects *in vivo* if indeed they exist. Relative to other *in vitro* bioassays, potential disadvantages include the need to develop and characterize recombinant cell lines, in some cases longer assay duration (6), and greater system complexity. Expression assays vary considerably in sensitivity, specificity, and responsiveness (in terms of fold induction), due to the wide variety of constructs used (6). This can make it difficult to compare results from different assay systems and laboratories.

Environmental Monitoring

In addition to the need to screen chemicals being produced and used, there is a need to monitor levels of estrogenic compounds in the environment. Monitoring is an essential part of the risk assessment process. It is also necessary to evaluate the need for and effectiveness of remediation and/or regulation efforts. Tools such as instrumental/analytical chemistry analyses and *in vivo* biomarkers, though not particularly suitable for screening applications, are useful for monitoring. Many of the *in vitro* methods discussed previously can be applied to monitor the environment for estrogenic compounds as well.

Instrumental analysis can be used to monitor the environment for known estrogen agonists or antagonists. Extracts can be prepared from water, sediments, soil, and biological matrices. The extracts can then be analyzed using gas chromatography (GC), high pressure liquid chromatography (HPLC), GC/mass spectrometry (GC/MS), etc. Such analysis can be very sensitive for common estrogen agonists, particularly due to the ability to concentrate samples during the extraction and clean-up process. The key advantage to this type of

analysis is the ability to precisely quantify concentrations of compounds of interest in the environmental matrix being studied.

Instrumental analyses alone, however, are rather limited as monitoring tools. Instrumental analyses, though quantitative, provide no information regarding the biological activity of the sample. Only compounds which have previously been identified as estrogen agonists or antagonists can be monitored in this fashion. This problem is further complicated by the fact that complex interactions may occur between various agonists, antagonists, and other compounds found in a complex environmental mixture. Analytical methods provide no mechanism by which to evaluate the overall activity of a mixture, unless strict additivity of effects is assumed. Non-target compounds in a complex environmental mixture may also interfere with proper resolution and quantification of target compounds. Thus, although instrumental analyses are powerful and sensitive tools, they cannot, alone provide the information needed for comprehensive monitoring and risk assessment.

Some of the *in vitro* bioassays used for screening are also useful for monitoring. Extracts from a variety of biotic and abiotic matrices can be tested. Unfortunately, as for instrumental analysis, the extraction and clean up process is often the most tedious and time consuming step in monitoring using *in vitro* bioassays. In some cases, however, less extensive sample preparation is necessary since the need to resolve individual compounds is not an issue for bioassays. The primary advantage to using *in vitro* bioassays for monitoring is that they should respond to any and all active compounds in the sample, not just a limited set of known or target compounds. Because of this, they can integrate the activity of the entire mixture, accounting for all potential interactions, whether they be additive or not.

One distinct advantage of monitoring with *in vitro* bioassays is the potential to derive and compare relative potencies. The concentration or volume of environmental extract needed to elicit a given level of effect can be compared to that of a standard, such as E₂, and expressed relative to it (*i.e.* E₂ equivalents = EC₅₀ sample / EC₅₀ E₂). This type of approach has been used extensively for dioxin-like compounds (144-147). It provides a simple method for comparing estrogenic potency from sample to sample, even when sample composition cannot be determined. Comparison of relative potencies of different fractions from the same sample can also be used to describe complex interactions that may be occurring in mixtures.

Unfortunately, however, it is not always easy or straight forward to calculate comparable potencies for environmental samples based on bioassay results. For comparison of potencies to be accurate and useful, the same response must be achieved with each sample. That is, the concentration associated with a particular magnitude of response needs to be estimated, and that magnitude must be the same for all samples being compared. This generally requires obtaining a complete dose-response for all samples being compared, or at least assuming that given a sufficient dose of sample, all will reach the same maximum level of response. In practice, however, these conditions are often difficult to meet and the assumptions do not usually hold. The inability to control the concentration of active compounds in the extract beyond the limits of sample concentration and/or dilution often precludes the ability to generate a complete dose-response curve such that maximal activity is achieved. Variation in the efficacy of different estrogen agonists within their range of solubility generally precludes the ability to assume that all samples will reach the same maxima (Fig. 3). This generally means that in order to estimate or compare potencies, the

concentration needed to elicit a minimum statistically significant response (LOEC) or threshold is about the only comparable point to use for all samples. This is not ideal, however, since there is minimal statistical confidence at this point.

This discussion illustrates the advantages and deficiencies of both instrumental and *in vitro* bioassay methods for monitoring estrogenic compounds in the environment. From the discussion, it should also be apparent, that both types of tools complement one another. Analytical and *in vitro* tools can be used effectively together to provide information needed for monitoring, as well as risk assessment, via bioassay-directed fractionation and identification. Such complementary application of instrumental and bioassay techniques is often referred to as *toxicant identification and evaluation* (TIE) (Fig. 6).

In the first step of a TIE scheme, samples from an abiotic or biotic matrix of interest are collected and chemicals are selectively extracted and fractionated, chromatographically, to separate the complex mixture from the matrix and into groups of compounds with nominal characteristics. In monitoring for the presence of estrogen agonists in water, samples of water could be passed through a solid phase sampling device, such as Sep-pacs or Empore™ disks. Alternatively, passive samplers such as semi-permeable membrane devices (SPMDs), can be placed in the environment to collect a time integrated sample of the materials of interest (148). Once a sample has been separated from its matrix it can be analyzed in an *in vitro* assay. This is generally a functional assay such as the MCF-7-luc assay used to test for estrogenicity. A battery of assays based on different functional end points could be used (52). If there were a positive response in the assay, the sample could be further fractionated based on

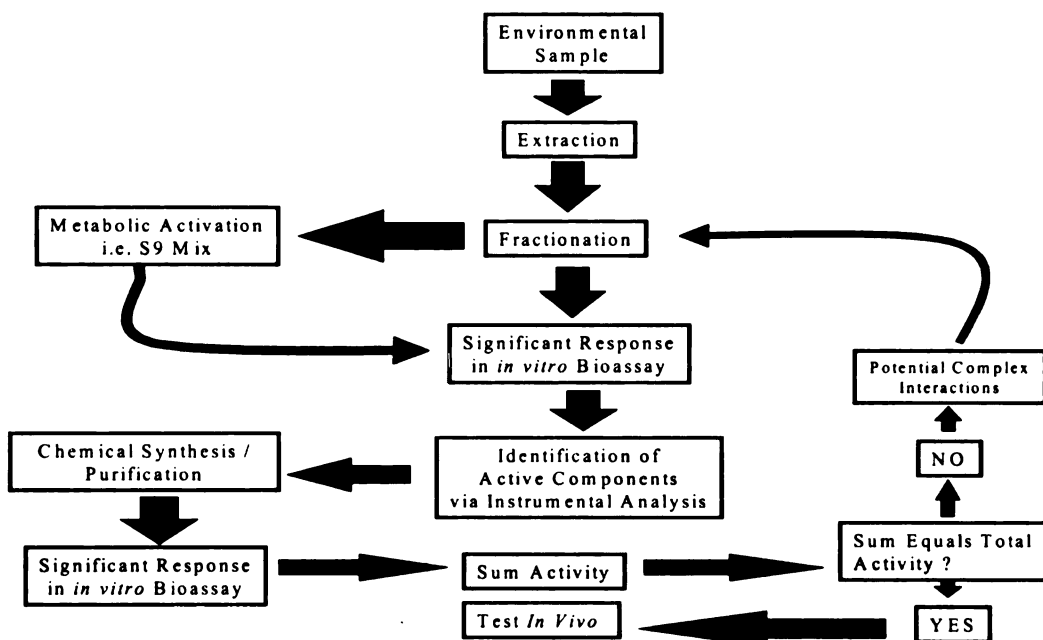


Fig. 6. Generalized toxicant identification and evaluation scheme for environmental samples using *in vitro* bioassay-directed fractionation and instrumental analysis.

molecular size, polarity or a combination of the two. Each fraction would then be subjected to bioassay. In this way the types of compounds contributing to a positive response could be narrowed to members of operationally-defined functional classes. Instrumental analyses would then be applied to the fraction(s) which elicited activity *in vitro*. Typically, active fractions are analyzed by several methods, such as HPLC with both fluorescence and UV-visible detection, gas chromatography with flame ionization detection (FID), electron capture detection (ECD), and/or mass-selective detection (MSD). The particular methods used depend on the properties of the fraction of interest. For instance, if the activity were in the non polar fraction, it could be analyzed by gas chromatography directly. However, if a more polar fraction contained the activity it might be necessary to use HPLC-based analysis or derivatization techniques. Additional separation of active fractions can be achieved using high pressure liquid chromatography (HPLC). Through additional iterations of fractionation, bioassay, and instrumental analysis, it should be possible to identify the specific bioactive agents present in the original extract. Once a tentative identification is achieved, standards would be used to quantify the mass of material in the sample. If authentic standards were not available commercially they can be synthesized. Pure compounds could then be analyzed by *in vitro* bioassay to determine if they are, indeed, active. If so, relative potency factors (RPFs) are derived and total equivalents determined for the sample by multiplying the RPFs by the molar concentrations of the compound in the sample. Predicted and measured activity in the assay are compared in a mass balance of potency. If they are equal, it indicates that all of the activity has been accounted for by the assay and that

there are no interactions among compounds. Alternatively there could be more activity or less activity in the measured or predicted values. More activity in the assay than predicted could indicate that there were additional unidentified compounds or superadditive interactions in the mixture. Less activity in the bioassay might indicate an infraadditive interaction among compounds. Use of selective isobolar additions and selective removal of compounds can confirm the presence or absence of interactions or unidentified compounds. A similar technique has been proposed for use to separate the relative contributions of endogenous and exogenous hormones in plasma (124).

In Vivo Biomarkers

In vivo biomarkers are also viable monitoring tools. Reports of *in vivo* vitellogenin induction (149), altered secondary sex characteristics (150,151,152) lowered sperm counts (44), etc. are responsible for much of the attention that endocrine disrupting compounds have drawn recently. These and other *in vivo* responses can be monitored as indicators of potential exposure to estrogenic and other endocrine disrupting compounds. Such indicators have the advantage of incorporating all the complex pharmacokinetic and metabolic factors that can affect uptake and disposition of compounds in a whole organism, giving them more biological significance. In some cases, however, it is not known whether the changes observed are adverse or not. This limits the risk assessment utility of some biomarkers. Because *in vivo* endpoints are influenced by so many additional variables, both physiological and environmental, it is much harder to establish clear cause effect relationships between responses and specific compounds in the environment. Thus, *in vivo* biomarkers are most

useful for monitoring and risk assessment as part of a comprehensive, tiered approach that incorporates both instrumental analyses and rapid *in vitro* screening assays.

For each receptor-mediated mechanism, an agonist/antagonist screen can potentially be developed. This can serve as the first tier in an integrated monitoring approach. *In vivo* models can provide a second tier for evaluating the toxicological significance of active samples. In conjunction with chemical analysis, this tiered approach could be used to monitor for and identify specific bioactive compounds in the environment and evaluate their potential toxicological relevance.

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Chapter 2

RAINBOW TROUT CELL BIOASSAY-DERIVED RELATIVE POTENCIES FOR HALOGENATED AROMATIC HYDROCARBONS: COMPARISON AND SENSITIVITY ANALYSIS

Daniel L. Villeneuve†, Catherine A. Richter‡, Alan L. Blankenship†, John P. Giesy†

† Department of Zoology, National Food Safety and Toxicology Center, and Institute for
Environmental Toxicology, Michigan State University, East Lansing, Michigan 48824,
USA

‡ Biochemistry Department, M121 Medical Sciences, University of Missouri-Columbia,
Columbia, Missouri 65212, USA

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Abstract-Rainbow trout hepatoma cells, stably transfected with a luciferase reporter gene under control of dioxin responsive elements (RLT 2.0 cells) were used to derive relative potencies (RPs) for a variety of halogenated aromatic hydrocarbons (HAHs) that are structurally similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This in vitro bioassay utilizes 96-well microplates which provides high sample throughput and assay efficiency without affecting sensitivity. RLT 2.0-derived potencies for dioxin and furan congeners, relative to 2,3,7,8-TCDD, ranged from 0.917 for 1,2,3,4,7,8-hexachlorodibenzofuran (HxCDF) to 0.208 for 1,2,3,7,8-pentachlorodibenzofuran (PeCDF). All mono- and di-ortho polychlorinated biphenyls (PCBs) tested had RPs which were orders of magnitude less than TCDD, but point estimates could not be determined. RLT 2.0-derived RPs were found to be comparable to both other rainbow trout-specific RPs and RPs based on mammalian bioassay. Sensitivity analysis suggested that the range of uncertainty associated with TCDD equivalents (TEQ) estimates based on RLT 2.0-derived RPs is approximately 10 fold. Within this degree of uncertainty and the context of this study, the RLT 2.0 bioassay showed no definitive biases or inaccuracies relative to similar mammalian- or fish-specific in vitro bioassays. Thus, the RLT 2.0 bioassay appears to be a useful tool for evaluating dioxin-like potency of HAHs to fish.

INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs) are a diverse group of chemicals which include polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs),

biphenyls (PCBs) and others. Some of these chemicals are ubiquitous, persistent, and toxic environmental contaminants [1]. These compounds have been shown to exert adverse effects on fish, including mortality, wasting syndrome, fin and gill lesions, hepatotoxicity, immunotoxicity, embryotoxicity, and reproductive impairment [2-5]. Such toxic effects have been observed at concentrations currently found in eggs of Great Lakes salmonids [6].

Rapid, sensitive, and economical methods for assessing the biological potency of environmental mixtures of HAHs are needed to facilitate risk assessment and formulate objective policy regarding HAHs. Instrumental analysis is difficult and expensive, and provides only limited information on the biological relevance of HAHs found in the environment [7]. Because HAHs are thought to exert many of their adverse effects through a common aryl hydrocarbon receptor (AhR)-mediated mechanism [1,8], in vitro bioassays which measure AhR-mediated enzyme induction have been used to screen for HAHs [7,9,10]. Correlations between AhR-mediated enzyme induction in vitro and toxic potency in vivo make such assays useful for estimating AhR mediated toxic potency of complex mixtures of HAHs [1,7]

Although it is a well established method, in vitro ethoxyresorufin-O-deethylase (EROD) assay with H4IIE rat hepatoma cells [9], has been criticized as not being entirely suitable for assessing the risk of AhR-active xenobiotics to fish and other aquatic organisms. Differences in sensitivity to AhR-active compounds between fish and mammals have been reported [4,11,12]. In particular, fish have been shown to be less sensitive to certain PCBs than mammals. As a result, a variety of both in vivo and in vitro approaches have been used to derive fish-specific relative potencies (RPs) [4,13,14].

Although they provide valid sets of fish-specific RPs, these methods are not particularly amenable to environmental monitoring and risk assessment of unknown compounds. In vivo approaches used to derive fish-specific RPs are costly and time consuming and thus not very useful as a rapid screening tool. In vitro EROD assays using fish cell lines such as RTL-W1 [13] and PLHC-1 [15,16] are more reasonable for this purpose since they are rapid, inexpensive, and sensitive fish-specific bioassays.

Another approach, developed recently, involves use of a novel recombinant rainbow trout cell line, RLT 2.0 [17]. RLT 2.0 cells are rainbow trout hepatoma cells stably transfected with a luciferase reporter gene under control of dioxin responsive elements (DREs) [10,17]. Binding of the AhR-ligand complex to the DREs results in an upregulation of luciferase transcription [17], which upon addition of a substrate, luciferin, catalyzes a light producing reaction. The luminescent end-point can be measured with a luminometer to provide a sensitive measure of AhR-mediated gene expression potency. Greater sensitivity, selectivity, and dynamic range have been cited as potential advantages of using luciferase-transfected cell lines rather than cytochrome P4501A1 (CYP1A1) expression in wild type cells as an endpoint [7]. Luciferase assays with recombinant H4IIE cells were demonstrated to be more sensitive than in vitro EROD assay using wild type H4IIE cells [7]. The RLT 2.0 bioassay was reported to have a detection limit of 4 pM TCDD [17], while no significant EROD activity was detectable in the parent cell line, RTH-149, at concentrations less than 100 pM TCDD [17]. Thus, the RLT 2.0 cell bioassay is fish (salmonid) specific and has advantages conferred by the reporter gene construct.

Historically, the terms toxic equivalency factor (TEF) and relative potency (RP) have been used synonymously. Most literature RP values cited in this paper are referred to as TEFs in the primary source. Recently, however, increasing efforts are being made to distinguish the two terms. In the context of this paper, RPs are defined as species-, endpoint-, and assay-specific determinations of potency expressed relative to some standard, such as TCDD. TEFs are defined as consensus values based on RP determinations across multiple species and/or endpoints. TEFs are commonly used for risk assessment purposes and are generally order of magnitude estimates. RPs are generally more precisely defined and are needed for bioassay directed mass balance analysis of complex mixtures.

The purpose of this study was to adapt the RLT 2.0 assay to a 96-well plate format and increase assay efficiency without significant loss of sensitivity or resolution. Such modifications should increase the utility of the assay for screening, and bioassay directed fractionation applications. In addition, a previously reported list of RLT 2.0-derived RPs [17] was expanded to aid mass-balance evaluations. New congeners tested included several mono- and di-ortho PCB congeners, 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), 1,2,3,7,8-pentachlorodibenzofuran (PeCDF), and 1,2,3,4,7,8-hexachlorodibenzofuran (HxCDF). RLT 2.0 derived RPs were compared to both fish-specific and mammalian RPs and TEFs commonly used in risk assessment and characterization of environmental samples. A sensitivity analysis was performed to characterize the uncertainty associated with application of RLT 2.0-derived values. Results presented should help guide the use of

RLT 2.0-derived potency to characterize environmental samples and evaluate aquatic exposure to HAHs.

MATERIALS AND METHODS

RLT 2.0 cell culture

RLT 2.0 cells were cultured in 100-mm disposable petri plates (Corning, Corning NY, USA) containing 12 ml Basal Medium Eagle (Life Technologies, Grand Island, NY, USA) supplemented with 10% defined fetal bovine serum (Hyclone, Logan UT, USA) and 292 mg/L L-glutamine (Sigma, St. Louis, MO, USA). Plates were incubated at 21°C in a 95:5 air:CO₂ (v/v) atmosphere with 80% relative humidity. Cells were passaged when plates became confluent (generally once per week). Every 5 passages, 1000 mg/L geneticin (G-418; Sigma G9516) was added to the culture medium to maintain selection pressure on the recombinant cells. New cultures were started from frozen stocks after 20 passages.

Chemicals and Reagents

2,3,7,8-TCDD, 1,2,3,4,7,8-HxCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, and PCBs 105, 118, 138, 153, 156, and 169 were purchased from Accustandard (New Haven, CT, USA). Working solutions and dilution series were prepared in pesticide residue analysis grade isooctane (Burdick & Jackson, Muskegon, MI, USA).

Luciferase assay reagent (LAR) was composed of 20 mM tricine (Life Technologies), 1.07 mM $\text{Mg}(\text{CO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ (Sigma), 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma), 0.1 mM EDTA-disodium salt (J.T. Baker, Phillipsburg, NJ, USA), 33.3 mM dithiothreitol (DTT; Sigma), 270 μM coenzyme A (Sigma), 530 μM ATP (Sigma), and 470 μM beetle luciferin (Promega, Madison, WI, USA). Viability reagent consisted of 1.0 μM calcein-AM (Molecular Probes, Eugene, OR, USA) and 1.0 μM ethidium bromide (Sigma) dissolved in non-supplemented Basal Medium Eagle.

Exposure of RLT 2.0 cells

RLT 2.0 cells, grown in medium without geneticin, were trypsinized from petri plates containing 80-100% confluent monolayers and resuspended in media before counting. Cell number per ml was estimated using a hemocytometer. Cells were diluted in medium to a concentration of approximately 7.5×10^4 cells/ml and seeded into the 60 interior wells of 96-well flat-bottom microplates (Corning 25860 for flash method as defined below, Packard Instruments 6005181 for glow method, Meriden, CT, USA) at 250 μl per well (15,000-20,000 cells per well) using a repeating pipettor. To assure homogeneity, the cell solution was vortexed continuously during seeding. The 36 exterior wells of each microplate were filled with 250 μl culture media. Cells were dosed after an overnight incubation to allow for cell attachment. Test and control wells were dosed with 2.5 μl of the appropriate test compound in isooctane or isooctane alone, respectively. Blank wells received no dose. Each plate tested included a minimum of three control wells, three blank wells, and one to three dilution series. Dilution series

consisted of six concentrations (3 replicate wells per concentration) of test compound. Dilution series ranged from 2-fold to logarithmic (10-fold). Dosed cells were exposed for 72 h at standard incubation conditions.

Luciferase assay

Luciferase assay methods used in this study were modifications of the previously published RLT 2.0 assay method [17]. The modifications were designed to adapt that method for use with a 96-well plate reading luminometer. Results from two methods are reported. The flash method was the first modification developed and most closely parallels the original assay method [17]. The glow method was developed to simplify the luciferase assay method to increase assay efficiency and sample throughput.

Luciferase assay: flash method

Culture medium was removed and each well was rinsed twice with phosphate buffered saline (PBS) using an eight channel vacuum manifold. 50 μ l PBS was added to each well and plates were inspected for cell loss due to washing. Following inspection, a viability assay was conducted [17]. 50 μ l of viability reagent was delivered to each well and plates were incubated for 10 min at room temperature. Fluorescence was measured with a Cytofluor 2300 (Millipore, Bedford, MA, USA) (excitation 485 and 530 nm, emission 530 and 645 nm). The viability solution was then removed by vacuum manifold and wells were rinsed three times with PBS. Plates were again inspected for

cell loss during washing, then lysed by addition of 30 μ l reporter lysis buffer (Promega) per well. After a 10 min incubation at room temperature, 20 μ l of lysate was transferred from each well of the transparent Corning microplate to the corresponding well of an opaque Microlite 2 plate (Dynatech Laboratories, Chantilly, VA, USA). The opaque plate was scanned using a ML 3000 microtiter plate reading luminometer (Dynatech Laboratories) set to enhanced flash mode. In this operation mode, 100 μ l LAR is injected directly into each well by an ML 3000 dispenser system (Dynatech Laboratories) while the well is directly under the photomultiplier tube. Peak light production and the time at which it occurs is recorded. Integration begins 5 s after LAR injection, with subsequent readings taken every 10 ms and integrated over 30 s before proceeding to the next well. Using this mode, total instrument run-time is approximately 35 min per plate.

A protein assay [18] was performed on the lysates remaining in the transparent Corning plate. This involved adding 90 μ l ultra pure water and 50 μ l 1.08 mM fluorescamine (Sigma) in acetonitrile to the 10 μ l lysate remaining in each well, incubating the plate for 15 min at room temperature, and scanning the plate with a Cytofluor 2300 (excitation 400 nm, emission 460 nm). A protein standard curve consisting of 6 concentrations of bovine serum albumin (BSA) (Sigma) ranging from 50-1.5 μ g per well, 4 replicates per concentration, was prepared and analyzed.

All data was collected electronically and imported into a spreadsheet program for analysis. Protein per μ l lysate was determined for each well by regression of relative fluorescence units (RFU) measured for that well against the BSA standard curve. Dose-response relationships using both unadjusted RLU (relative luminescence units) and RLU adjusted for protein (RLU/ μ g) versus log-dose were plotted. In general, correcting for

protein concentration did not significantly affect the variability of the data or the shape of the dose-response relationships. Only unadjusted data was used in deriving the results reported herein. A viability index (calcein fluorescence/ethidium fluorescence) was calculated for each well and this data was inspected for gross differences in viability between wells. Wells with a significantly different viability index were not included in data analysis. In general, significant differences in this ratio were observed only where differences in cell number were apparent by simple visual inspection of the wells.

Luciferase assay: glow method

Each test plate was inspected visually and differences in cell numbers and condition relative to control wells and conditions normally observed during routine culture were noted for each well. Culture medium was then removed and each well was rinsed twice with phosphate buffered saline (PBS) using an eight channel vacuum manifold. Plates were inspected for cell loss during washing. Cells were lysed for 5 min, at room temperature, with reporter lysis buffer (25 μ l per well) (Promega). After lysis, 75 μ l LAR was added to each well. Each plate was incubated for 10 min at 30° C then scanned with an ML 3000 microplate reading luminometer (Dynatech Laboratories) set to cycle mode, 4 cycles, 2 s pause between cycles. In this mode of operation, LAR is added to all wells prior to inserting the plate into the instrument. Relative light output of each well is scanned 4 times (4 cycles) and mean response, standard deviation, and coefficient of variation (CV) over the 4 cycles are reported for each well. Total run-time in the instrument is approximately 2 min per plate. Following the luminometer scan 125

μl 1.08 mM fluorescamine in acetonitrile was added to each well and plates were assayed for protein after a 15 min incubation at room temperature. Plates were scanned using a Cytofluor 2300 (excitation 400 nm, emission 460 nm) and responses were compared to a standard curve similar to that used for the flash method, but with appropriate volumes of glow method reagents.

All data was collected electronically and imported into a spreadsheet program for analysis. Protein content per well was calculated by regression against the BSA standard curve. Dose-response relationships depicting mean RLU (over three replicate wells) versus log-dose were prepared. Protein data was used as an index of cell number to detect outliers that were not apparent by visual inspection, but individual responses were not adjusted for protein.

Calculation of RPs

Responses expressed as mean RLU (3 replicate wells) were converted to a percentage of the mean maximum response observed for TCDD standard curves generated on the same day (%-TCDD-max). This was done to normalize responses for normal day-to-day variability in response magnitude. Responses expressed as %-TCDD-max were converted to probits, such that 50 %-TCDD-max yielded a probit value of 5.00. Probit units were plotted against log dose and EC-10s (probit = 3.718), 20s (probit = 4.159), and 50s (probit = 5.00), were calculated based on regression through the straight-line portion of each probit plot. Mean EC-10s (not reported), 20s, and 50s, were calculated for replicate dose-response relationships for each compound. RPs were

calculated for 1,2,3,4,7,8-HxCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, and 1,2,3,4,7,8-HxCDF as mean EC-20 and EC-50 values for TCDD / mean EC-20 and EC-50 values for the test compound.

The probit method used for estimating absolute and relative potencies in this study is a parallel line method which assumes the following conditions: 1) all compounds tested have equal efficacy (maximum response) if tested at sufficiently great concentration; and 2) the dose-responses being compared are parallel (have equal slopes). Not all dose-responses being compared in this study met these conditions. Replicate curves for TCDD and 1,2,3,4,7,8-HxCDD (Fig. 1-a,c) represent the ideal situation for use of a parallel lines method. Approximately equal efficacy was apparent and the slopes were nearly equal. Replicate curves for 1,2,3,7,8-PeCDF (Fig. 1-b) represent the worst-case situation encountered in this study. The efficacy observed for 1,2,3,7,8-PeCDF was less than 50% that of TCDD (Fig. 1-b). Thus, the first condition was violated. The slopes were reasonably similar considering the variation in observed efficacy, but differences may cause some inaccuracy in potency estimates. Although assumptions were violated in a few cases, reasonable parallel line approximations of relative potency were attainable for most dose-responses generated in this study. Over 90% of the dose-responses generated included responses $\geq$ 50%-TCDD-max. and all included values $\geq$ 20%-TCDD-max. Thus, it was unnecessary to extrapolate beyond the data to achieve potency estimates. Inaccuracies caused by violation of assumptions may impact the variability of some estimates and subsequent uncertainty, but should not significantly alter study conclusions.

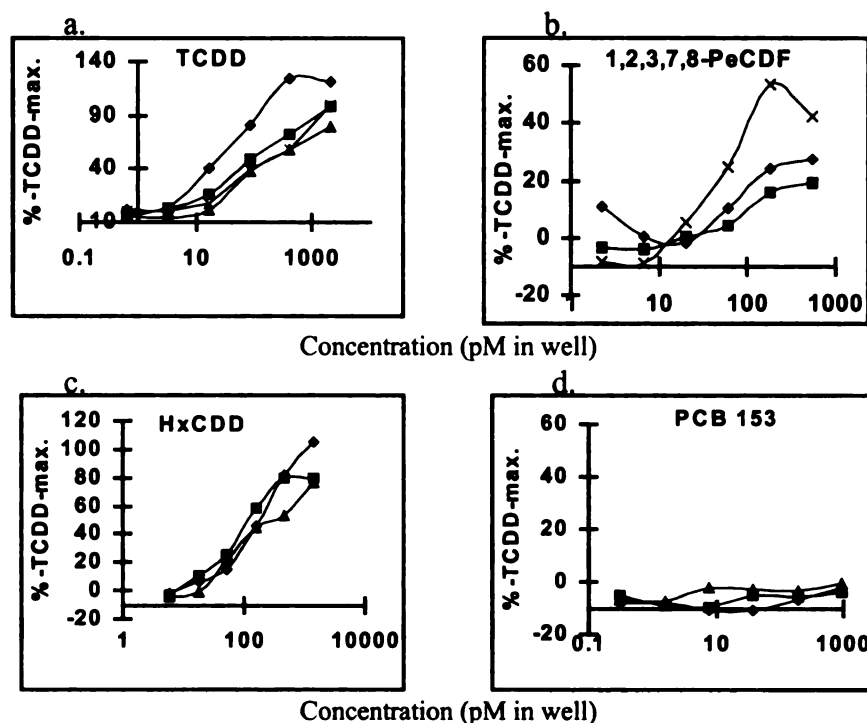


Fig 1. Examples of replicate dose-response curves generated using the RLT 2.0 bioassay (glow method). (a.) Replicate 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard curves ($n = 4$). (b.) Replicate dose-responses for 1,2,3,7,8-pentachlorodibenzofuran (PeCDF) ($n = 3$). Worst-case encountered for application of probit analysis for estimating EC-20s and EC-50s. (c.) Replicate dose-responses for 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD) ($n = 3$). (d.) Example of replicate dose-responses obtained for mono- and di-*ortho* PCB congeners ($n=3$). No responses were significantly different from background. Note, negative values for percentage of the mean maximum response observed for TCDD standard curves generated on the same day (%-TCDD-max) are due to subtraction of mean solvent control response prior to calculation of %-TCDD-max.

Sensitivity Analysis

Replicate RP determinations for TCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 1,2,3,4,7,8-HxCDD, and 1,2,3,4,7,8-HxCDF were fit to a selection of continuous frequency distributions (normal, log-normal, and uniform) using Crystal Ball[®] (Decisioneering, Boulder, CO, USA). Chi square goodness of fit was determined for each distribution. A log-normal distribution provided the best fit for all congeners except 2,3,7,8-TCDF which was best described by a normal distribution. Distributions were defined for all congeners using the arithmetic mean and standard deviation of all replicate RP determinations. Where replicate determinations were not available (congeners tested in a previous study [17], and PCBs) log-normal distributions with a mean and standard deviation equal to the value reported (or maximum possible RP for PCBs) were assigned. The RP of each congener was varied independently over its frequency distribution for 2000 separate trials and a TEQ for each sample was calculated for each trial as $TEQ_j = \sum (RP_{ij} * Conc._i)$, where i represents a different congener and j represents one of $n = 2000$ trials. A sensitivity analysis was performed on each frequency distribution of TEQs generated in this manner in order to determine the relative contribution of each RP to the total variation in the TEQ estimate.

RESULTS

RLT 2.0-specific RPs

None of the PCB congeners tested in this study were active in the RLT 2.0 assay system. Finite RPs for PCBs 77 and 126 (Table 1), both non-ortho substituted congeners, were reported previously [17]. PCBs tested in this study were predominantly mono- and di-ortho substituted congeners. Dose-response relationships were not obtained for any of the PCB congeners tested in this study and none of the concentrations tested produced a response significantly different from background (Fig. 1-d). Consequently, point estimates of RP could not be calculated for these compounds. RPs were reported as less than the value obtained by dividing the maximum concentration tested by the mean EC-20 or EC-50 for TCDD (Table 1). Actual relative potency values for these mono- and di-ortho PCB congeners are probably much lower than the values presented. Not all congeners were tested to their limit of solubility, consequently it may still be possible to generate a response in the cell line and calculate a point RP estimate for some of these congeners.

Point estimates of RPs were generated for all dioxin and furan congeners tested (Table 1). All congeners had potencies similar to that of 2,3,7,8-TCDD. Their approximate order of potency in RLT 2.0 cells was 1,2,3,4,7,8-HxCDF $\approx$ 2,3,7,8-TCDD > 1,2,3,4,7,8-HxCDD >> 1,2,3,7,8-PeCDF $\approx$ 2,3,4,7,8-PeCDF $\approx$ 1,2,3,7,8-PeCDD $\approx$ 2,3,7,8-TCDF. RPs based on results from the flash method were lower than those generated from glow method results. There was no consistent relationship in the relative

Table 1. RLT 2.0 bioassay-derived relative potencies (RPs) for halogenated aromatic hydrocarbons presented for two different bioassay methods. RPs based on both EC-50s and EC-20s are presented.

Compound ^a	Flash Method (EC50 RP)	Glow Method (EC50 RP)	Flash Method (EC20 RP)	Glow Method (EC20 RP)
2,3,7,8-TCDD	1.0	1.0	1.0	1.0
1,2,3,7,8-PeCDD ^b	0.225		0.279	
1,2,3,4,7,8-HxCDD	0.781	0.743	0.411	0.877
2,3,7,8-TCDF	0.228	0.243	0.0970	0.361
1,2,3,7,8-PeCDF	0.112	0.304	0.0582	0.287
2,3,4,7,8-PeCDF ^b	0.296	—	0.347	
1,2,3,4,7,8-HxCDF	0.735	1.10	0.328	1.04
PCB 77 ^{b,c}	0.00595	—	0.00173	—
PCB 105 ^d	<0.00005	—	<0.000006	—
PCB 118 ^d	<0.006	—	<0.0007	—
PCB 126 ^c	0.00628	—	0.00717	—
PCB 138 ^d	<0.036	—	<0.004	—
PCB 153	—	<0.00015	—	<0.00004
PCB 156 ^d	<0.036	—	<0.004	—
PCB 169	<0.005	—	<0.0006	—

^a TCDD = tetrachlorodibenzo-*p*-dioxin; PeCDD = pentachlorodibenzo-*p*-dioxin; HxCDD = hexachlorodibenzo-*p*-dioxin; TCDF = tetrachlorodibenzofuran; PeCDF = pentachlorodibenzofuran; HxCDF = hexachlorodibenzofuran.

^b Reported previously [17].

^c Estimated from incomplete dose-response curves.

^d Showed no response in flash method bioassay. Not analyzed using glow method.

magnitude of the EC-20 versus EC-50 based RP estimates. The absolute difference between the EC-20-RP and EC-50-RP tended to be less for the glow method. The coefficient of variation (CV) across the four values reported for each congener in Table 1 was greatest for 1,2,3,7,8-PeCDF (65%) and least for 1,2,3,4,7,8-HxCDD (29%). The average CV across the four RP values for each congener presented in Table 1 was 46%.

EC-50 and EC-20 estimates derived for the active compounds tested (Table 2) provide additional information that is not readily apparent from RP values alone. EC-50s and 20s based on the glow method were approximately half the magnitude of those based on flash method. Estimates based on the glow method were also less variable, with an average CV of 71% for EC-50s and 47% for EC-20s compared to 103% and 72% for the flash method. Neither the difference in magnitude nor the difference variability was statistically significant ($p = 0.05$, two-tailed), however. EC-20 estimates were less variable than EC-50 estimates.

DISCUSSION

Comparison of methods

As part of this study, two methods for performing the RLT 2.0 bioassay were developed and tested. Both methods were designed for use with a 96-well plate reading

Table 2. RLT 2.0 bioassay-derived potency of halogenated aromatic hydrocarbons. EC50 and EC20 estimates (pM in well) generated using flash and glow methods are presented. Estimates are the mean of *n* replicates $\pm$ one standard deviation (SD).

Compound ^a	Flash Method EC50 (SD, <i>n</i>)	Flash method EC20 (SD, <i>n</i>)	Glow method EC50 (SD, <i>n</i>)	Glow method EC20 (SD, <i>n</i>)
2,3,7,8-TCDD	299 (435, 14)	34.5 (31.6, 14)	147 (115, 4)	38.9 (20.6, 3)
2,3,7,8-TCDF	1310 (980, 5)	355 (204, 5)	603 (192, 3)	108 (23.5, 3)
1,2,3,7,8-PeCDF	2650 (2320, 5)	593 (321, 5)	1860 (2760, 3)	321 (377, 3)
1,2,3,4,7,8-HxCDF	406 (392, 4)	105 (70.4, 4)	133 (66.7, 3)	29.4 (7.13, 3)
1,2,3,4,7,8-HxCDD	382 (420, 5)	83.8 (74.5, 5)	197 (89.6, 3)	44.4 (9.48, 3)

^a TCDD = tetrachlorodibenzo-*p*-dioxin; TCDF = tetrachlorodibenzofuran; PeCDF = pentachlorodibenzofuran; HxCDF = hexachlorodibenzofuran; HxCDD = hexachlorodibenzo-*p*-dioxin.

luminometer and thus have significant advantages over the original assay method [17] in terms of sample throughput and assay efficiency. Results generated using the 96-well plate methods were more variable than results generated using the original method [17]. Using the method published previously [17], the average CV across EC-50 estimates for TCDD standard curves was 12.4% (n=4). The average CV across EC-50 estimates for TCDD standard curves was 146% (n=14) and 78.5% (n=4) for the flash and glow methods, respectively. Replicate to replicate variability (among replicate wells on a plate, or replicate determinations with cuvet reading luminometer [17]) was similar for all methods, however. Average CVs among replicates were 15.24%, 17.35%, and 15.6%, for glow, flash, and original [17] methods, respectively. The increased variability among standard curves (indicated by greater variability in EC-50 determinations) may be due to changes in the stability cell line and its response to HAHs, or differences in the method. The instrumentation needed to conduct a direct comparison of the methods was not available, therefore, the source of the increased variability could not be definitively determined. Assuming differences in the method are the cause, the 96-well plate methods remain effective for screening large numbers of samples, but the RLT 2.0 assay method published previously [17] may be more suitable for precise determination of relative potencies.

The glow method appears to be a more suitable method for use in routine bioassays than the flash method. The glow method greatly increases assay efficiency and sample throughput. Using the glow method, the limiting factor on the number of plates that can be analyzed on a given day is the time it takes to dose the plates (3 d prior to

analysis). One person can dose 10 to 20 plates in 8 h. Analysis of ten plates (3 d later) can be accomplished in less than 2 h. It is possible to analyze over 50 plates per instrument in a single 8 h day. Using the flash method, it takes an entire 8 h day to analyze 10 plates. Thus instrumental analysis-time becomes the limiting factor on the number of samples a laboratory can process.

In addition to decreasing instrument analysis-time, the glow method greatly streamlined the bioassay procedure and subsequent data analysis. Experience with the flash method indicated that performing a viability assay prior to the luciferase assay did not increase the quality of bioassay results. A simple visual inspection of each well on the test plates, prior to analysis, provided a more informative and equally reliable method for detecting potential cytotoxicity or contamination that could yield spurious data. Elimination of the viability assay from the procedure reduces the number of washing steps during which cell loss can occur. The need for lysate transfer was eliminated in the glow method by using opaque 96-well plates with a transparent bottom. Wells could be inspected visually throughout the exposure period, then a sticker could be applied to the bottom of the plate to render it opaque for luciferase assay, and subsequently removed to facilitate a protein assay. Elimination of the lysate transfer step was advantageous since it was a step where errors in technique could easily generate considerable variability and/or inaccurate results. It is reasonable to assume that the flash method could benefit from similar changes in procedure.

Glow method simplifications were not detrimental to assay sensitivity or variability. The average variability between replicate wells on a single plate and variability between replicate dose-response curves was less for the glow method,

although the difference in average CVs between methods was not statistically significant ($p = 0.05$, two-tailed) in either case. Mean EC-50s and EC-20s for the glow method were, on average, 47% less than estimates based on the flash method. This suggests that the glow method may be slightly more sensitive than the flash method. Again, this difference was not statistically significant, however ($p = 0.05$, two-tailed). Thus, the glow method represents a significant improvement in assay efficiency without loss of sensitivity and resolving power.

Comparison of relative potencies

RLT 2.0 RPs were reasonably well correlated with early life stage mortality (ELSM) RPs but poorly correlated with in vivo and in vitro EROD RPs in rainbow trout ($R^2 = -0.34$ and -0.07 , respectively; Table 3). The greatest correlation between any of the rainbow trout specific RPs was between in vitro and in vivo EROD induction ($R^2 = 0.83$; Table 3). RPs based on ELSM were most closely correlated with World Health Organization (WHO) TEFs [19] for fish ($R^2 = 0.95$; Table 3). This is probably because WHO fish-TEFs were largely based on ELSM-RPs [19]. The lack of strong correlation (Table 3) between the various sets of rainbow trout specific RPs suggest that within the range of uncertainty associated with these assays, there is no consensus rank order of potency for the dioxins, furans, and non-ortho PCBs among rainbow trout specific bioassays.

In terms of magnitude, RLT 2.0 derived RPs compare favorably with other sets of rainbow trout-specific RPs (Table 3). Within the range of uncertainty, there were

essentially no differences between rainbow trout RPs for TCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,4,7,8-HxCDF and PCB congeners (Table 3). No differences greater than 10-fold were observed between RPs for any specific compound, despite the range of endpoints encompassed.

RLT 2.0-RPs for 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF were approximately 10-fold greater than both EROD based RP estimates (Table 3). They were relatively close to ELSM-RPs, however. This may suggest that RLT 2.0 may be a better model for predicting the in vivo potency of these compounds than those based on CYP1A1 induction.

RLT 2.0-RPs for 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF were similar to EROD based RPs but are approximately 10-fold greater than ELSM-RPs (Table 3). There is some evidence to suggest metabolic inactivation and/or excretion in vivo may account for such a difference. Half-lives reported for 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF in mammals and fish in vivo are shorter than those for 2,3,7,8-TCDD, 1,2,3,4,7,8-HxCDD, and 2,3,4,7,8-PeCDF in both fish and mammals (only mammalian half-lives were available for 1,2,3,7,8-PeCDF) [20]. This points out a potential limitation of RLT 2.0 and EROD based assays for determining the potency of PCDFs to rainbow trout.

Overall, the RLT 2.0 bioassay appears to be a reasonable method for screening samples for potential to cause dioxin-like biological responses in rainbow trout. RLT 2.0 RPs may slightly over or under-estimate the potency of individual congeners but no definitive biases or inaccuracies were apparent based on comparison to other rainbow trout RPs.

Table 3. Comparison of RLT 2.0 bioassay derived relative potencies (RPs) to rainbow trout-specific RPs based on other in vitro and in vivo endpoints and World Health Organization (WHO) toxic equivalency factors (TEFs) for fish. A correlation matrix is presented. Correlations were calculated using only those congeners for which point estimates of RP were available for both columns being compared.^a

Compound	RLT 2.0	In vitro EROD ^b	In vivo EROD ^c	In vivo ELSM ^d	WHO TEF ^e
2,3,7,8 TCDD	1.0	1.0	1.0	1.0	1
1,2,3,7,8 PeCDD	0.225 ^f	2.6	1.8	0.73	1
1,2,3,4,7,8 HxCDD	0.762 ^g	1.1	0.4	0.319	0.5
2,3,7,8 TCDF	0.235 ^g	0.2	0.5	0.028	0.05
1,2,3,7,8 PeCDF	0.208 ^g	0.2	0.4	0.034	0.05
2,3,4,7,8 PeCDF	0.296 ^f	1.9	2.0	0.359	0.5
1,2,3,4,7,8 HxCDF	0.917 ^g	1.1	0.4	0.280	0.1
PCB 77 (3,3',4,4')	5.95 x 10 ^{-3 f,h}	--	--	0.00016	0.0001
PCB 105 (2,3,3',4,4')	<5x10 ^{-5 g}	--	--	<7x10 ⁻⁵	<5x10 ⁻⁶
PCB 118 (2,3'4,4',5)	<6x10 ^{-3 g}	--	--	<7x10 ⁻⁵	<5x10 ⁻⁶
PCB 126 (3,3',4,4',5)	6.28x10 ^{-3 f}	--	--	0.005	0.005
Correlation Matrix	R ²	R ²	R ²	R ²	R ²
RLT 2.0	1.0	-0.072	-0.34	0.61	0.45
<i>In vitro</i> EROD		1.0	0.83	0.58	0.70
<i>In vivo</i> EROD			1.0	0.49	0.63
<i>In vivo</i> ELSM				1.0	0.95

^a EROD = ethoxyresorufin *O*-deethylase; ELSM = early life stage mortality; WHO = World Health Organization; TEF = toxic equivalency factor; TCDD = tetrachlorodibenzo-*p*-dioxin; PeCDD = pentachlorodibenzo-*p*-dioxin; HxCDD = hexachlorodibenzo-*p*-dioxin; TCDF = tetrachlorodibenzofuran; PeCDF = pentachlorodibenzofuran; HxCDF = hexachlorodibenzofuran.

^b [13] ^c [14] ^d [4] ^e [19] ^f [17] ^g this study

^f EC-50 estimates based on incomplete dose-response curves

RLT 2.0-RPs are similar to RPs generated using a mammalian in vitro bioassay (H4IIE rat hepatoma cells) (Table 4). RLT 2.0-RPs were similar to the limited set of H4IIE-luc (recombinant H4IIE cells) -RPs reported in the literature (Table 4). They were also approximately equal to H4IIE-wt (wild type) -RPs for TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-TCDF, and 1,2,3,7,8 PeCDF (Table 4). The RLT 2.0-RP for 2,3,4,7,8-PeCDF was nearly five-fold less than the corresponding H4IIE-wt estimate (Table 4). It was similar to H4IIE-luc-RPs and international TEFs for this congener, however. This suggests that, the H4IIE-wt value may be an overestimate. The H4IIE-wt value for 2,3,4,7,8-PeCDF (Table 4) agrees with EROD based estimates in rainbow trout (Table 3). Thus, the difference may be endpoint-dependent such that EROD assays may overestimate the in vivo potency of this compound. The greatest differences between H4IIE and RLT 2.0 RPs were observed for 1,2,3,4,7,8-HxCDD, and 1,2,3,4,7,8-HxCDF (Table 4). RLT 2.0-RPs were 9- and 45-fold greater, respectively. All rainbow trout-RPs (Table 3) were at least 4-fold greater than H4IIE-wt estimates (Table 4). The differences suggest that endpoints in fish and fish cell lines, may be more responsive to hexachlorinated PCDD and PCDF congeners than mammalian cells.

Due to the inability to generate point estimates of RP for the mono- and di-ortho PCBs tested in this study, it is not yet clear whether the RLT 2.0 cell line is a better model for predicting the in vivo potency of these compounds to fish. Differences in

Table 4. Comparison of RLT 2.0 bioassay derived relative potencies (RPs) to RPs based on *in vitro* bioassay with H4IIE-rat liver cells (recombinant [luc] and wildtype [wt]) and international toxic equivalency factors (TEFs).

Compound ^a	RLT 2.0 RP	H4IIE-luc ^b RP	H4IIE-wt ^c RP	International TEFs ^c
2,3,7,8 TCDD	1.0	1.0	1.0	1.0
1,2,3,7,8 PeCDD	0.225 ^d	0.79	0.420	0.5
1,2,3,4,7,8 HxCDD	0.762 ^e	-- --	.0830	0.1
2,3,7,8 TCDF	0.235 ^e	-- --	0.200	0.1
1,2,3,7,8 PeCDF	0.208 ^e	-- --	0.200	0.05
2,3,4,7,8 PeCDF	0.296 ^d	0.69	1.40	0.5
1,2,3,4,7,8 HxCDF	0.917 ^e	-- --	0.020	0.1
PCB 77 (3,3',4,4')	5.95 x 10 ^{-3 d,f}	0.00071	1.8 x 10 ⁻⁵	5 x 10 ⁻⁴
PCB 105 (2,3,3',4,4')	< 5 x 10 ^{-5 e}	<1x10 ⁻⁶	8 x 10 ⁻⁶	1 x 10 ⁻⁴
PCB 118 (2,3'4,4',5)	< 6 x 10 ^{-3 e}	<1x10 ⁻⁶	3.5 x 10 ⁻⁷	1 x 10 ⁻⁴
PCB 126 (3,3',4,4',5)	6.28 x 10 ^{-3 d}	0.017	2.2 x 10 ⁻²	0.1
PCB 156 (2,3,3',4,4',5)	< 3.6 x 10 ^{-2 e}	-- --	5.5 x 10 ⁻⁵	5 x 10 ⁻⁴
PCB 169 (3,3',4,4',5,5')	< 5 x 10 ^{-3 e}	0.00055	4.7 x 10 ⁻⁴	1 x 10 ⁻²

^a TCDD = tetrachlorodibenzo-*p*-dioxin; PeCDD = pentachlorodibenzo-*p*-dioxin; HxCDD = hexachlorodibenzo-*p*-dioxin; TCDF = tetrachlorodibenzofuran; PeCDF = pentachlorodibenzofuran; HxCDF = hexachlorodibenzofuran.

^b [7] ^c [21] ^d[17] ^e this study

^f EC-50 estimates based on incomplete dose response curves

responsiveness of mammals and fish to mono-ortho PCBs have been reported [4,11,16]. This supports the hypothesis that a fish-specific in vitro bioassay model should be more accurate in predicting the in vivo potency of samples containing mono-ortho PCBs to fish, than a mammalian in vitro bioassay.

Sensitivity analysis

A common use for RPs and TEFs is in deriving a single value to characterize the biological potency of a sample based on instrumental analysis. This is generally done by multiplying the concentration of each compound detected by its RP or TEF and summing the total for all compounds. The value attained is known as a TCDD equivalent (TEQ). TEQs can be used to characterize potential biological potency based on instrumental analysis alone or they can be applied in conjunction with bioassay. TEQs calculated for risk assessment purposes tend to be based on TEFs. TEQs used in a mass balance context, in conjunction with bioassay, tend to be based on assay specific RPs. Comparison of RP-TEQs with bioassay derived TCDD-equivalents (TCDD-EQs) provides a method to evaluate mass balance and possible interactions between compounds. In the case of an unknown sample, if bioassay-derived TCDD-EQs are approximately equal to a calculated TEQ, one can assume that the active compounds in the sample have been detected and accounted for. Large differences between TCDD-EQs and TEQs suggest either non-additive interactions between compounds or failure to detect and account for all active compounds in the sample. Bioassay directed

fractionation and further instrumental analysis can be applied to elucidate which of the above circumstances apply.

A sensitivity analysis was conducted to determine the uncertainty contributed to assessment of complex environmental mixtures of HAHs by uncertainties in RPs determined for individual congeners and by differences in RPs between in vitro and in vivo responses and among species. The sensitivity analysis was conducted by calculating a set of TEQs from representative sets of RPs and TEFs. Instrumental analyses reported for samples of three species of fish (carp, walleye, and alewife) collected from Saginaw Bay, MI, USA [22] were used [22-Tables 6-10, small walleye, alewife, carp]. Congener concentrations and relative distributions in these samples should be representative of fish, from a variety of trophic levels, exposed to HAHs in situ.

Sensitivity analysis was used to determine which RLT 2.0-RPs are likely to contribute most to uncertainty in RLT 2.0-derived TEQs. A frequency distribution of TEQs was generated for each species (Fig. 2) and a sensitivity analysis was performed to determine the relative contribution of each RP to the total variation in the TEQ estimate (Table 5). Values within the TEQ distributions for carp, walleye, and alewife varied up to 15-fold (Fig. 2). Within the 95% confidence range, carp and alewife TEQs varied up to six-fold and walleye TEQs varied up to eight-fold. This suggests that a ten-fold uncertainty factor ($\pm$ five-fold) is probably appropriate for TEQs derived from the current set of RLT 2.0-RPs.

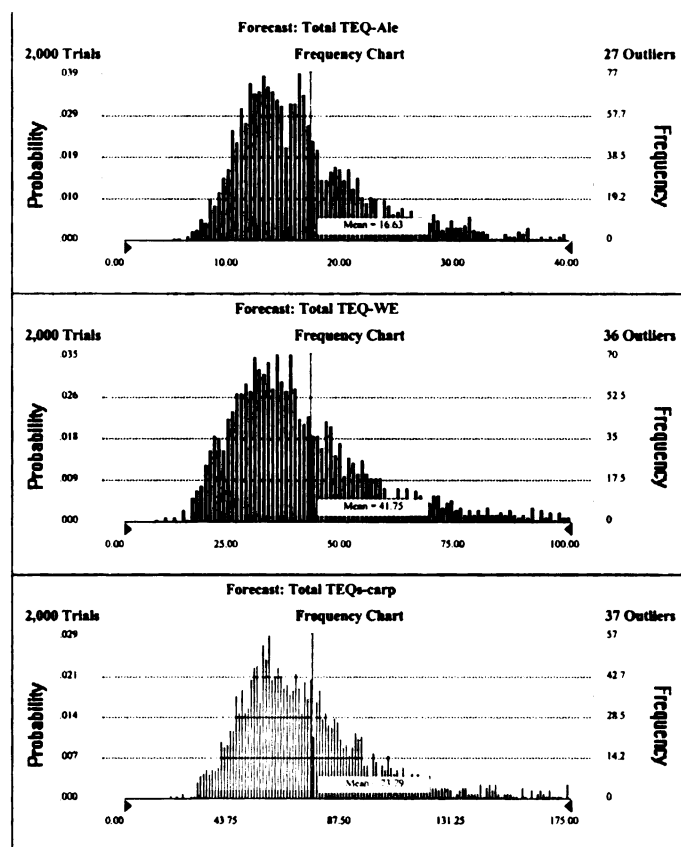


Fig. 2. Frequency distributions for alewife (**top**), walleye (**middle**), and carp (**bottom**) tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs) generated by Monte Carlo Simulation ($n = 2000$ independent trials). RLT 2.0-relative potencies (RPs) for all compounds listed in Table 1 were allowed to vary independently over empirically defined, congener specific, frequency distributions.

Table 5. Sensitivity analysis: percent contribution to total variance in tetrachlorodibenzo-*p*-dioxin (TCDD) equivalent (TEQs), calculated for three separate samples, caused by congener specific uncertainties in RLT 2.0 relative potency (RP) estimates. Determined by Monte Carlo Simulation ($n = 2000$ independent trials). RLT 2.0-RPs for all congeners listed in Table 1 were allowed to vary independently over empirically defined, congener specific, frequency distributions^a.

Carp-TEQ ^b		Walleye-TEQ ^b		Alewife-TEQ ^b	
Congener	% ^c	Congener	% ^c	Congener	% ^c
2,3,7,8 TCDD	46	PCB 77	74	PCB 77	61
2,3,4,7,8 PeCDF	24	2,3,7,8 TCDF	13	2,3,7,8 TCDD	14
2,3,7,8 TCDF	13	2,3,7,8 TCDD	7.5	PCB 126	10
PCB 138	3.5	PCB 126	2.5	2,3,7,8 TCDF	6.5
1,2,3,7,8 PeCDF	3.5	PCB 138	1.0	PCB 138	3.5
All others	10	All others	2	All others	5

^a PeCDF = pentachlorodibenzofuran; TCDF = tetrachlorodibenzofuran.

^b Based on instrumental analysis of Saginaw Bay fish tissue samples [22].

^c Refers to % contribution to total TEQ variance due to uncertainty in the RP estimate for that congener.

Dioxin and furan congeners contributed most of the uncertainty to carp-TEQs, while walleye and alewife TEQs were more sensitive to non-ortho PCBs (Table 5). In carp, RPs for TCDD, TCDF, and PCDF were the major source of variability in the TEQ estimate, with TCDD accounting for 46% of the variance (Table 5). Walleye- and alewife-TEQs were more sensitive to uncertainty in the RPs for PCB 77 and 126 (Table 5). TEQs for all three species were sensitive to the RP estimate for PCB 138. This suggests that simply using the maximum possible RP for this congener could lead to significant overestimation of potency. This could confound mass balance analyses based on such an estimate. Consequently, the RP of PCB 138 should be determined more precisely. Even at their maximum possible RPs no other mono- or di-ortho PCBs contributed significantly to the calculated concentrations of TEQ. This suggests that use of the maximum limit for these congeners may not significantly bias the TEQ estimates for fish with a similar exposure/ accumulation profile. Overall, the sensitivity analysis suggests that more precise estimates of RP for TCDD, PCB 77, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, PCB 126, and PCB 138, in that order, would best decrease the uncertainty of RLT 2.0 based TEQ estimates for similar samples.

Sensitivity of TEQ estimates to in vitro, in vivo, and between species differences in reported RPs was evaluated. RLT 2.0, H4IIE-wt, ELSM, and international-TEQs were calculated for carp, walleye, and alewife using the values presented in tables 3 and 4. In order to avoid uncertainty associated with RP estimates for mono- and di-ortho PCB congeners, TEQs were based solely on dioxin [22-Table 9], and furan [22-Table 10]

congeners presented in table 3, and PCBs 77 and 126 [22-Table 8]. The resulting TEQs are presented (Fig. 3).

Within the range of certainty determined for RLT 2.0-TEQs there were no statistically significant differences between RLT 2.0-TEQs and those generated using other sets of RPs. TEQs based on RLT 2.0 bioassay were, on average, 50% greater than those based on in vivo early life stage mortality in fish. H4IIE and international TEQs were, on average, 140%, and 250% greater than ELSM-TEQs, respectively. All values were within the same order of magnitude and all four sets of TEQs were significantly correlated with one another ($R^2 = 0.755-0.998$). These results suggest that, based on dioxin, furan, and non-*ortho* PCB congeners, RLT 2.0-based characterization of a complex mixture would yield essentially the same conclusions as in vivo ELSM characterization despite absolute differences in RP estimates (Table 3) discussed previously. Furthermore, RLT 2.0 characterization would also be comparable to that based on H4IIE-wt RPs and international TEFs. Relatively large differences in the RPs for specific congeners like 1,2,3,4,7,8-HxCDF and -HxCDD between RLT 2.0 and mammalian RPs did not result in equally large differences in TEQs. These results support the hypothesis that the RLT 2.0 bioassay is a valid method for evaluating the potency of AhR-active compounds. They do not, however, support the hypothesis that a fish-specific cell line is necessary to estimate in vivo potency of dioxins, furans, and non-*ortho* PCBs to fish.

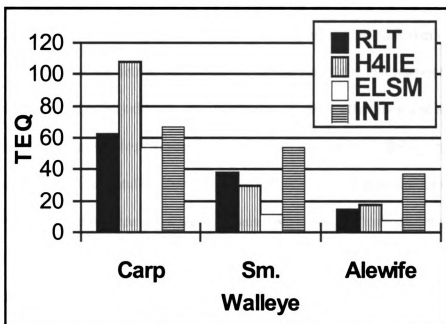


Fig. 3. Comparison of tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQ) estimates based on instrumental analyses of Saginaw Bay carp, walleye, and alewife samples [22], calculated using RLT 2.0 (RLT), H4IIE-wild type (H4IIE), or *in vivo* rainbow trout early life stage mortality (ELSM) relative potencies or international toxic equivalency factors (INT) (Tables 3,4). TEQ estimates shown here are based on concentrations of PCBs 77 and 126, and the dioxin and furan congeners listed in Table 1.

Conclusions

RLT 2.0 in vitro bioassay is a useful tool for characterizing AhR mediated biological potency, comparable to other assays currently used for this purpose. The original RLT 2.0 assay method [17] was modified to increase efficiency and sample throughput without marked loss in precision or sensitivity. RLT 2.0-RPs were reported for a number of environmentally relevant HAHs but precise RP estimates for mono- and di- ortho PCB congeners were not achieved. Sensitivity analysis indicated that RLT 2.0-RPs could be applied with about 10 fold uncertainty to calculate TEQs based on instrumental analysis. This is comparable with the uncertainty around most TEFs used for risk assessment purposes. The utility of RLT 2.0-RPs could be increased by more precise determinations of RPs for specific congeners identified by sensitivity analysis. For dioxins, furans, and non-ortho-PCBs, RLT 2.0 bioassay does not appear to be more accurate than analogous mammalian bioassays (like H4IIE assays) for estimating in vivo potency in fish. The results presented do not support or reject the hypothesis that RLT 2.0 bioassay may more accurately predict the in vivo potency of mono- and/or di-ortho PCB congeners, to fish, than in vitro bioassay with mammalian cells, however. Further studies are needed to test this hypothesis.

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Chapter 3

DERIVATION AND APPLICATION OF RELATIVE POTENCY ESTIMATES BASED ON IN VITRO BIOASSAY RESULTS

Daniel L. Villeneuve*, Alan L. Blankenship, and John P. Giesy

**Dept. of Zoology, National Food Safety and Toxicology Center, and Institute for
Environmental Toxicology, Michigan State University, East Lansing, MI, USA, 48824**

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Abstract- Relative potency (RP) estimates are widely used to characterize and compare the potency of a wide variety of samples analyzed using in vitro bioassays. The most commonly reported RP estimate is a single ratio of potency estimates calculated at a defined level of response such as the EC-50. RPs based on a single ratio of point estimates are only valid when the slope and efficacy (maximum achievable response) of the sample are equivalent to those of the standard to which they are being compared. Often, these conditions are either violated or cannot be demonstrated. As a result, there is a need to calculate and present relative potencies in a manner which addresses the potential uncertainties caused by violation of the assumptions of parallel slopes and equal efficacy. Uncertainty due to non-parallel slopes can be evaluated mathematically, but uncertainty due to efficacy differences cannot. Use of multiple point estimates to derive ranges of RP estimates, termed RP-bands, is recommended. RP-bands can provide a discrete characterization of relative potency without sacrificing accuracy. Furthermore, they provide a means to test the assumption of parallel slopes in situations where statistical tests are not applicable. A systematic method for evaluating sample efficacy has been developed into a framework to guide the derivation and application of RP estimates based on in vitro bioassay results. Use of the systematic framework and multiple point estimates was illustrated using three sample data sets. It is hoped that the framework and discussion presented will facilitate the use of bioassay-derived RP estimates to characterize samples of both known and unknown composition without sacrificing accuracy.

INTRODUCTION

In vitro bioassays can be useful tools for environmental monitoring. They provide rapid and cost-effective methods to screen large numbers of samples for their ability to elicit a biological response through a specific mechanism of action. Though instrumental analyses are essential for the identification and quantitation of compounds in complex environmental mixtures, instrumental results are not well suited for predicting and/or understanding potential effects of complex environmental mixtures on biota [1]. Instrumental analyses can miss compounds that are biologically active at concentrations below analytical detection limits or compounds for which there are no established methods or analytical standards [1]. Furthermore, even when compounds are detected, instrumental analyses provide no information regarding their biological potencies, particularly in conjunction with the other components of the mixture. In vitro bioassays can integrate the overall potency of samples containing complex mixtures of compounds inactive compounds, agonists, and antagonists which may be interacting both additively and non-additively. Because they are based on biologically relevant mechanisms of action, in vitro bioassays can provide an indicator of the mechanism-specific, biological potency of a sample.

To facilitate quantitative risk assessment and simplify data interpretation, a complex mixture's potency to cause a defined biological response is often expressed relative to that of a well-characterized standard or prototypical compound (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] and 17- β -estradiol [E₂]) [2-4]]. In theory, expression of sample potency in terms of equivalents of a standard compound allows for

comparison of diverse samples and may provide a basis for approximating risk to biological organisms with analogous biochemical processes [1,2]. Additionally, bioassay-derived equivalents can be compared to equivalents calculated from congener-specific instrumental analyses and established toxic equivalency factors (TEFs) or assay specific relative potencies (REPs) in a mass balance analysis [1,5-7]. This kind of analysis can be used to assess whether the known composition of a sample (based on instrumental analyses), can account for the bioassay responses observed [1,5-10]. Lack of agreement between bioassay-derived and instrumentally-based equivalents can suggest either the presence of unidentified, biologically active, compounds in a sample, or non-additive interactions between components of the sample [1,5-10]. Thus when coupled with chemical fractionation and instrumental analysis, in vitro bioassays become very powerful tools for characterizing environmental samples when accurate relative potency estimates can be derived.

Relative potency estimation

Relative potency estimation or the determination of TCDD- or E₂-equivalents (-EQ) can be considered a type of indirect bioassay. An indirect bioassay has been described as one in which an estimate of equally effective doses of standard and sample is determined, such that the inverse ratio of their equally effective doses describes the potency of the sample relative to a standard [11]. The underlying assumption of an indirect assay is that doses of standard and sample which yield some selected magnitude of response in a bioassay can be defined as equally effective doses [11]. Thus, at any

given magnitude of response, the potency of a sample relative to a standard is the ratio of the doses of standard and sample needed to elicit that response [11] (Equation 1).

$$\text{Relative potency} = \text{dose std}_i / \text{dose sample}_i$$

(1)

where i = a defined magnitude of response

The concentration needed to elicit a 50% response is widely regarded in toxicology as the standard point estimate for describing the potency of a chemical to elicit a specified response in an organism or test system [12]. EC-50s and other point estimates (EC_x) can be calculated using a wide variety of methods including probit or logit analysis [12-14], Scatchard and Woolf analysis [15,16], graphical interpolation, linear regression, and non-linear regression [17,18]. As a result, relative potencies have traditionally been calculated as the ratio of the EC-50s of the two compounds being compared (Equation 2).

$$\text{relative potency} = \text{EC-50}_A / \text{EC-50}_B$$

(2)

typically where A= a well characterized standard compound and B= the test compound

Although this is a logical approach which seems compatible with the nature of indirect bioassay, relative potencies based on a ratio of point estimates are only valid under very limited conditions [11,14,19,20]. Indirect bioassay assumes that the sample

being analyzed responds as if it were a dilution (or more concentrated form) of the standard compound [11]. This implies that the dose-response curves being compared are parallel (Fig. 1A) [11,14,19]. They should be effectively identical except for their position along the x-axis when log dose is plotted against response (Fig. 1A) [20]. For these conditions to be met, the dose-responses must have a common slope [11,14,19] and the maximum achievable response (efficacy) for the standard and sample must be identical [20] (Fig. 1A).

For non-parallel dose-response relationships (Fig. 1B) relative potency is a function of dose [11,20]. The relationship described at a single level of response, such as the EC-50, is not constant over the entire range of responses for the compounds being compared [14] (Fig. 1B). Relative potency estimates based on a ratio of EC-20s, EC-50s, and EC-80s, for example, would be quite different (Fig. 1B). Thus, a point estimate of relative potency may only represent a single point along a broad range of potential relative potencies. Use of a single point estimate can lead to misleading and/or inaccurate interpretations. When dose responses cross in the positive quadrant even the rank order of potency may change [20]. Thus, it has been argued that point estimates of relative potency do not provide an accurate characterization of relative potency for compounds whose dose-responses are not statistically parallel [14,20]. Parallelism should be demonstrated before a relative potency estimate is calculated [11,14,19].

Statistical methods for testing parallelism are available [11,14,19]. Such tests are of little value in attempting to compare complex mixtures or unknowns to a standard compound, however. Due to their complex or unknown composition environmental samples and other unknowns cannot be assigned a meaningful set of dose units which can

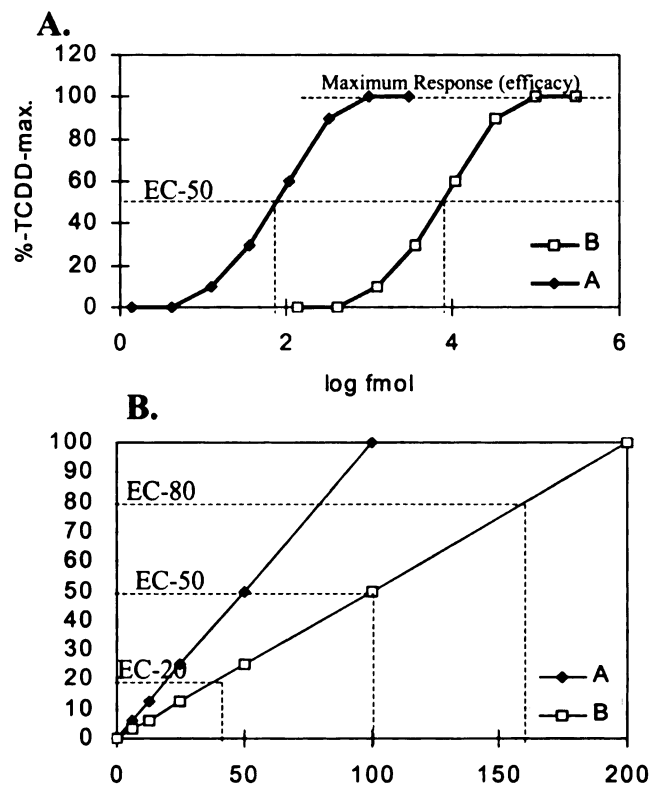


Fig. 1. A. Illustration of relative potency estimation for dose response relationships which conform to the assumptions of indirect bioassay (i.e. equal efficacy and parallel slopes). Relative potency is constant over the effective response range. B. Illustration of relative potency estimation for dose-response relationships which do not conform to the assumption of parallel slopes. Relative potency varies over the effective response range.

be statistically compared to those of the standard compound. Even if they can be plotted on a common scale such as volume of extract, mass of sample, percent dilution, etc., a statistical test for parallelism is not biologically meaningful. As a result, statistical tests for parallelism cannot be applied to unknowns and complex mixtures.

Furthermore, there is little reason to assume that dose-response relationships for complex mixtures analyzed by the same in vitro bioassay will be parallel or show equal efficacy. Even for single compounds of known concentrations, effects such as partial agonism, differences in binding affinities, in vitro toxicokinetics, solubility, sensitivity to environmental conditions, etc. can produce non-parallel dose-responses and limit the magnitude of response that can be achieved [20]. In a complex mixture, the likelihood of violating the assumptions of indirect bioassay is greatly increased. Interactions between compounds in the sample could be expected to produce variations in the shape of the dose-response relationship. For example, as the concentration of agonists in the sample increases, so too might the concentration of antagonists, such that a more gradual slope and lower maximum response is produced. Alternatively, compounds in a complex mixture may affect other aspects of cell function which cross-talk with the pathway of interest to modulate the magnitude and/or rate of change in response relative to dose [21]. Furthermore, for environmental samples, lack of control over sample composition, limited aqueous solubility, limited sample volume, and the need to conserve sample for other analyses can often limit the ability to achieve a maximal level of response. As a result, sample efficacy is often unknown. Such factors could be expected to produce significant variation in dose-response relationships from sample to sample or relative to a

standard. Thus, parallelism and equal efficacy can neither be assumed nor, in the case of parallelism, demonstrated statistically, for complex mixtures and unknowns.

While numerous authors have discussed the problems associated with the use of point estimates to characterize relative potencies, there remains a need to reduce complex dose-response data to simple quantitative estimates, in order to simplify data interpretation and risk assessment. The purpose of this paper was to develop a framework to facilitate the use of bioassay-derived relative potency estimates to characterize samples of known and unknown composition without sacrificing accuracy of data interpretation.

Relative potency estimation methods which incorporate multiple point estimates (MPE) are recommended as an alternative to single point estimation techniques in situations where adherence to the assumptions of indirect bioassay cannot be demonstrated. These estimates are presented in a manner which identifies uncertainties in the relative potencies derived. A systematic framework for evaluating in vitro bioassay results was developed to facilitate evaluation of indirect bioassay assumptions, direct selection of appropriate relative potency estimation techniques, and guide the use and interpretation of the estimates generated. Application of MPE methods and use of the systematic framework is demonstrated using several example data sets.

METHODS

Multiple Point Estimates

In order to be meaningful for risk assessment, relative potency estimates must be representative of the relationship between sample and standard over their entire effective range of response. The problem with non-parallel dose-responses is that a single ratio of point estimates does not provide a characterization that is representative of all positions along the curves. Relative potency estimates will vary with the response level selected [11,14,20] (Fig. 1B). Estimates based on the 50% level of response (EC-50) may give very different results from those based on the 20% or 80% response [14,20,22] (Fig. 1B). One solution to the problem stated above would be to report relative potencies as a function, rather than a quantitative estimate [20]. Although more accurate, this would tend to make risk assessment and data interpretation a rather cumbersome and laborious process. As an alternative, when the dose-responses being compared are non-parallel or cannot be demonstrated to be parallel, statistically, relative potency can be determined for multiple points along the effective range of responses. The range of relative potency values generated, which we have termed a relative potency band (RP-band), can provide useful information about the dose-responses being compared without sacrificing accuracy.

MPE techniques provide an empirical method for testing the parallel slopes assumption of indirect bioassay. For parallel curves, RP is independent of response (Fig. 1A) (or dose), thus, the RP-band for parallel curves should be, essentially, a single value.

The wider the RP-band, the greater the curves deviate from parallelism. Thus, coupled with empirical observations of sample efficacy, the calculation of an RP-band allows the assumptions of indirect bioassay to be evaluated for complex mixtures and unknowns.

MPE methods also provide a means to derive useful and accurate relative potency information for non-parallel dose-responses. RP-bands provide a measure of the potential range of uncertainty in relative potency values that is generated by selection of the response level at which a point estimate is taken (uncertainty due to non-parallel slopes). As long as the sample and standard dose-responses have the same efficacy and have been modeled over the entire range of effective response, the RP-band generated provides a quantitative estimate of relative potency that is valuable for comparing among samples as well as establishing TEFs or RPs, conducting mass balance analyses, guiding risk assessments, etc. The only limitation is whether or not the range of uncertainty in the RP estimate is sufficiently small for the desired application. For example, an RP-band which ranges from 1-10 pg TCDD-EQ/g sample may be useful for risk assessment purposes, but may not be sufficient for mass balance analysis or comparing it's potency to that of similar samples.

One limitation to the RP-band approach is that the width of the band is sensitive to the range of responses selected. As a result, in order for RP-bands to be directly comparable and give an independent measure of the uncertainty due to non-parallel slopes, it is necessary to standardize the range of response over which they are calculated. This standard range has arbitrarily been defined as 20%-80% of the maximum response achieved for the standard compound (20-80%-std.-max.). For samples with different or unknown efficacies, it may be necessary to extrapolate beyond the known range of

response in order to calculate an RP-band over this range. In such cases, the uncertainties involved in such an extrapolation should be reported and discussed.

Relative Potency Bands

RP-bands are fairly simple to calculate. The first step involves fitting an appropriate regression model to the dose-response data. Once a regression model has been defined for each of the dose-responses being compared, the regression equation for each curve is used to solve for the doses associated with multiple levels of response (Y_i) over a standard range (20-80%-std.-max.) and equation 1 is used to calculate a relative potency value (RP_i) for each level of response (Y_i) selected. The maximum and minimum RP_i values define the limits (or range) of the RP-band (Equation 3).

$$RP\text{-band} = \text{minimum } RP_i \text{ to maximum } RP_i \quad (\text{Equation 3})$$

where RP_i = the relative potency determined at a defined level of response Y_i
and multiple values for Y_i are tested such that they encompass the standardized range of 20-80%-std.-max..

The simplest approach for calculating the RP-band involves selecting response levels (Y_i) which correspond to 20%-, 50%-, and 80%-std.-max. and calculating an RP_i value for each response value (Y_i). In situations where slopes vary over the standardized range of response, Monte-Carlo simulation may be used to calculate RP_i over a uniform distribution whose limits are defined as 20-80%-std.-max. Using this approach RP_i can

be calculated rapidly for hundreds of points along the standardized range of response, and the maximum and minimum RP_i generated can be reported. This can be done using computer programs such as Crystal Ball® (Decisioneering, Boulder, CO, USA). The principle is the same, only the number of response levels (Y_i) tested differs. For cases in which equal efficacy cannot be demonstrated, one of the points (Y_i) selected should be the response level corresponding to the maximum response observed for the sample in question. The RP_i determined at this point should be identified when presenting the RP-band, and all RP_i calculated at responses greater than the maximum observed (extrapolated RP_i) should be highlighted to indicate that they were based on extrapolation beyond the range of empirical data.

Interpretive framework

A systematic framework to guide derivation, critical evaluation, and use of relative potency estimates based on in vitro bioassay results was developed (Fig. 2). The framework was designed as a dichotomous decision tree that guides critical evaluation of the dose-responses being compared. A series of yes/no questions regarding the properties of the dose-responses guides the user to a reasonable method for calculating and interpreting relative potency using MPE methods.

Fit regression model (Fig. 2-A). The first step in any attempt to estimate relative potency is to fit an appropriate regression model to the dose-response relationship. Dose

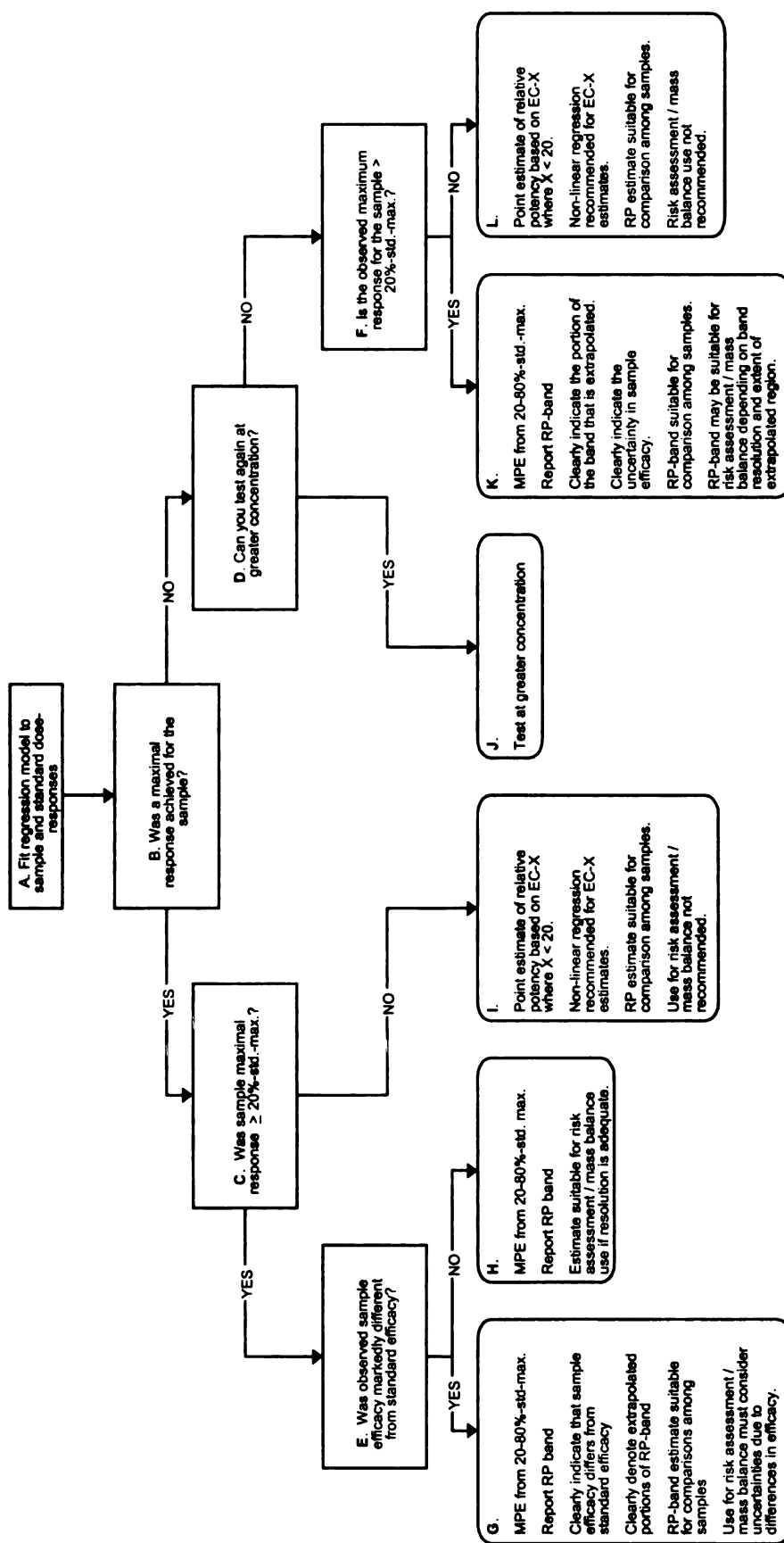


Fig. 2. Systematic framework to guide derivation and application of relative potency (RP) estimates from in vitro bioassay data using multiple point estimates (MPE). RP band = the range of relative potency values derived over a standard range of responses (20-80%-std.-max.). %-std.-max. = sample response expressed as a percentage of the maximum response magnitude achieved for the standard compound.

responses from in vitro bioassay will generally not be linear when plotted in the original dose and response units. In some cases, however, a linear transformation can be used to simplify data analysis without biasing the conclusions [11]. Commonly used transformations include log-dose, probit, logit, and logistic [11,14,18,23-25]. In addition to linear transformations and linear regression, non-linear models may be applied to characterize dose-response relationships [17,18,24,25]. In particular, generalized linear models and other non-linear regression techniques have been recommended for modeling relatively low responses since they utilize the inherent S shape of the dose-response relationship [18,22,24-26]. Choice of an appropriate model to describe a concentration-response curve is often arbitrary, but may be quite critical, particularly when the estimation technique involves extrapolation from the observed data [22]. Readers are directed to the references provided above for detailed descriptions on fitting regression models to dose response data.

Evaluate Sample Efficacy. For indirect bioassay to be valid, the samples and standard being compared must have the same efficacy [11,20]. The assumption of equal efficacy can be tested empirically by examining whether the maximum responses observed for the samples and standard were approximately equal (or statistically equal if replicate dose-responses are available). This requires that the maximal response of the sample is known (Fig. 2-B). For the purpose of this discussion a maximal response is defined as the magnitude of response at the point where the S-shaped dose-response curve reaches the upper plateau.

When a maximal response is achieved, the assumption of equal efficacy can be evaluated (proceed to Fig. 2-C). If the maximal response of the sample is less than 20%-std.-max. the assumption of equal efficacy is clearly violated and MPE will generally not be suitable for deriving a reasonable RP estimate (Fig. 2-I). If necessary, a point estimate of RP can be made at a level of response less than 20%-std.-max., but such an estimate is not suitable for risk assessment or mass balance purposes (Fig. 2-I). Non-linear regression methods are recommended for deriving point estimates of RP at response levels less than 20%-std.-max. (Fig. 2-I). Such estimates may be cautiously applied for comparing among samples (Fig. 2-I), but the marked differences in efficacy between sample and standard should be clearly noted. If the maximal response of the sample is greater than 20%-std.-max., it is generally feasible to derive at least an approximate estimate of relative potency using MPE methods (proceed to Fig. 2-E). If replicate dose-response curves are available, statistical methods may be used to determine whether the observed efficacy of the sample and standard are statistically equivalent (Fig. 2-E). In cases where the observed maximal response for the sample and standard differ markedly (Fig. 2-G), MPE methods can still be employed to derive an approximate estimate of sample RP. Such estimates are not completely valid, however. Differences in efficacy, and extrapolation beyond the empirical range of sample response should be clearly noted and considered when using the approximations for comparative, risk assessment, or mass balance purposes (Fig. 2-G). In cases where the observed maximum response of the sample and standard do not differ markedly, RP-bands provide a valid quantitative estimate of relative potency (Fig. 2-H). Such estimates are suitable for risk assessment or

mass balance applications, provided the width of the RP-band and the resultant range of uncertainty in the estimate is small enough to provide adequate resolution.

If a maximal response was not achieved, equal efficacy cannot be demonstrated empirically or tested statistically (proceed to Fig. 2-D). If possible, the sample should be tested at greater concentrations (Fig. 2-J). In some cases, this may not be feasible, however, due to logistical constraints. In situations where the maximal response of the sample is unknown, a RP estimate can be derived, but the uncertainties due to unknown efficacy must be considered (proceed to Fig. 2-F). If the observed maximum response for the sample is greater than 20%-std.-max., MPE methods can be used to calculate an RP-band approximation of the sample RP (Fig. 2-G). Again, the extrapolated region of the band and potential uncertainty due to unknown efficacy must be identified and discussed when presenting and applying the estimates (Fig. 2-G). If the observed maximum response of the sample is less than 20%-std.-max., use of MPE to calculate an RP-band is not feasible (Fig. 2-H). If an estimate is needed, non-linear regression may be used to derive a point estimate of RP at a level of response less than 20%-std.-max (Fig. 1-L). Such an estimate should be interpreted and applied conservatively, however, due to the inability to evaluate the assumptions of indirect bioassay.

Example data sets

Several example data sets were used to demonstrate the use of the systematic framework (Fig. 2) and RP-bands to evaluate relative potency based on in vitro bioassay

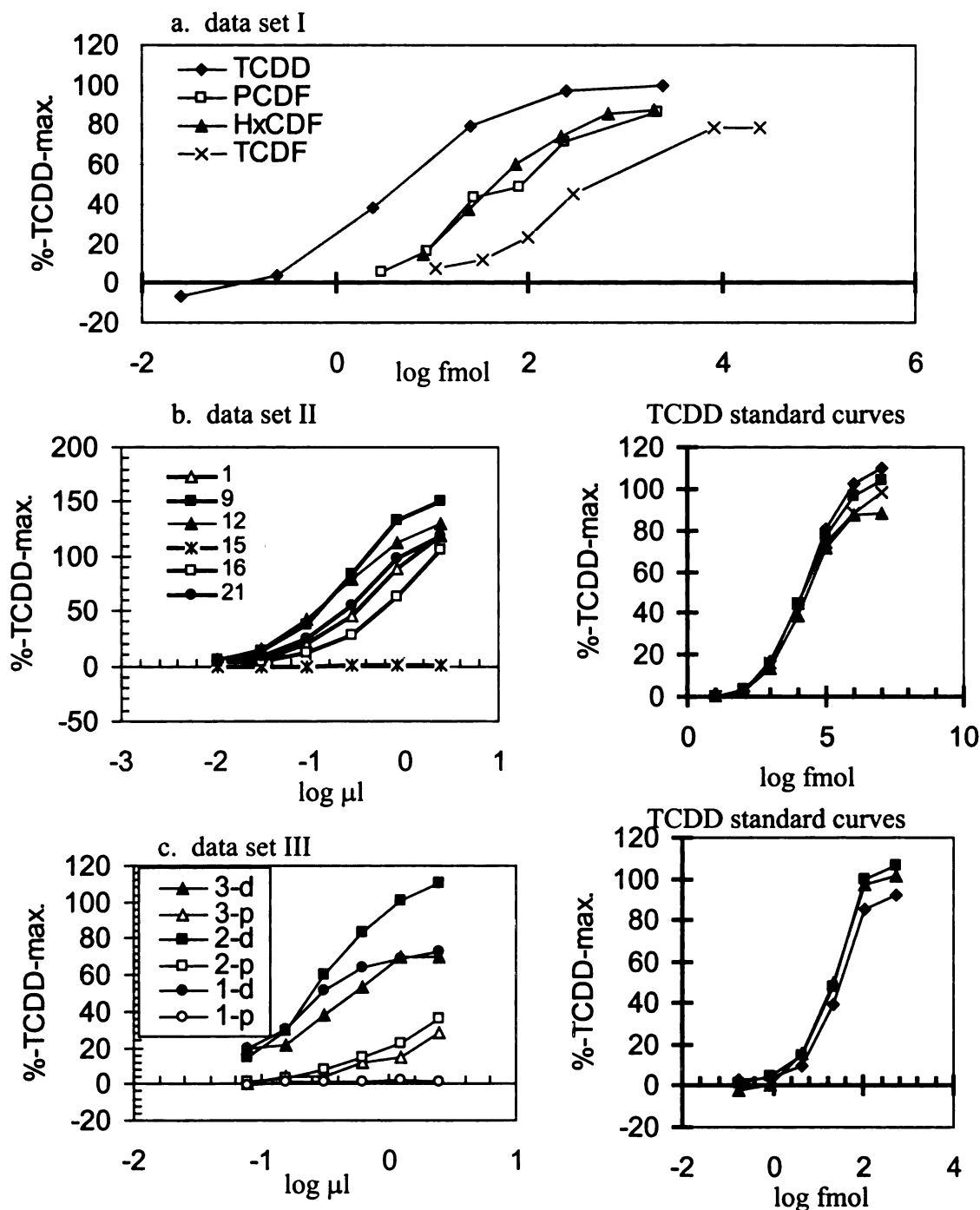


Fig. 3. Example data sets. (a.) data set I - H4IIE-luc responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzofuran (PCDF), 1,2,3,4,7,8-hexachlorodibenzofuran (HxCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF). (b.) data set II - H4IIE-luc responses to extracts of sediment from Masan Bay, Korea and corresponding TCDD standard curves. (c.) data set III - RLT 2.0 responses to extracts of sediment collected from a superfund site contaminated with Aroclor 1268 and corresponding TCDD standard curves. Response magnitudes presented as a percentage of the mean maximum response observed for the corresponding TCDD standard (%-TCDD-max.)

results. Example data sets I (Fig. 3a) and II (Fig. 3b) represent the response of H4IIE-luc cells [27] to individual halogenated aromatic hydrocarbons (HAHs) and sediment extracts, respectively. H4IIE-luc cells are rat hepatoma cells stably transfected with a luciferase reporter gene under control of dioxin responsive enhancers (DREs) [27]. In vitro H4IIE-luciferase assays were conducted as described previously [10,28]. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzofuran (PCDF), 1,2,3,4,7,8-hexachlorodibenzofuran (HxCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) analyzed for data set I (Fig. 3a) were purchased from Accustandard (New Haven, CT, USA). Working solutions and dilution series were prepared in pesticide residue analysis grade isooctane (Burdick and Jackson, Muskegon, MI, USA). Dose responses for PCDF and HxCDF consisted of six 3-fold dilutions, with the maximum concentration being equal to 8.8 and 8.0 nM for PCDF and HxCDF, respectively. TCDF was tested at the following six concentrations: 98, 33, 1.2, 0.40, 0.13, and 0.045 nM. Sample dose response curves were compared to a TCDD standard curve (10-fold dilution series, 6 concentrations, 10-0.0001 nM). Sediment extracts (data set II) were prepared from sediments collected from Masan Bay, Korea. Sample collection, extraction, fractionation, and analysis was performed using methods detailed elsewhere [28,29]. Dose-responses consisted of six 3-fold dilutions of each extract or fraction. Sample dose-response curves were compared to a TCDD standard curve (5-fold dilution series, 6 concentrations, 0.67 - 2100 pM).

Example data set III (Fig. 3c) was generated using the RLT 2.0 bioassay which utilizes rainbow trout hepatoma cells transfected with a luciferase reporter gene under control of DREs to screen for Ah-receptor-active compounds [30,31]. Extracts from

sediments collected from a superfund site contaminated with Aroclor 1268 were analyzed. Detailed methods for the collection, preparation, fractionation, and instrumental analysis of the sediment samples were reported elsewhere [32-34]. RLT 2.0 bioassay was performed according to the glow method presented by Villeneuve et al. [31] which is a modification of a procedure presented by Richter et al. [30]. Dose-responses consisted of six 3-fold dilutions of each extract or fraction. Sample dose-response curves were compared to a TCDD standard curve (5-fold dilution series, 6 concentrations, 0.67 - 2100 pM).

Data analysis

Example data sets I, II, and III were analyzed using the systematic framework outlined above. Sample responses expressed in relative luminescence units (RLU) were converted to a percentage of the mean maximum response observed for the TCDD standard (%-TCDD-max.). The mean solvent control response was subtracted from both sample and standard responses, prior to conversion to a percentage, in order to scale the values from 0 to 100%-std.-max. Responses expressed as %-TCDD-max. were plotted as a function of either log μ l (sediment extracts) or log fmol (standards and known compounds) (Fig. 3). For simplicity, the linear portion of each example dose response was defined by dropping points from the tails until a $R^2 \geq 0.95$ was obtained and a linear regression model was fit. Although this technique may not be appropriate for all situations, particularly for evaluation of relative potency at low levels of response, it was sufficient for the purposes of this paper. Regression equations for the samples and

standards were then used to calculate RP_i values for $Y_i = 20, 50, \text{ and } 80\% \text{-TCDD-max.}$ (Equation 1). For samples whose observed maximum response were less than 80%-TCDD-max. an additional RP_i estimate was calculated at $Y_i = \text{observed maximum response expressed as } \% \text{-TCDD-max.}$ RP-bands were then generated based on the multiple RP_i point estimates. For comparative purposes, RP-bands were also calculated by Monte Carlo analysis (2000 iterations, uniform response distribution ranging from 20-80%-TCDD-max.) using Crystal Ball[®] (Decisioneering). For all the example data sets used, Monte Carlo analysis gave the same RP-band as the simple 3-point or 4-point calculation.

RESULTS and DISCUSSION

Applying the Framework

Data set I. A linear regression model was fit to each dose-response curve (Fig. 2-A). In this case, the samples consisted of single compounds of known concentration and three replicate dose-response curves were available for each compound tested. As a result, statistical methods could be used to test the assumptions of indirect bioassay. Maximal responses were achieved for all samples (Fig. 2-B; Fig. 3a) and all those responses were greater than 20%-TCDD-max (Fig. 2-C; Fig. 3a). Sample efficacy was not statistically different from that of the TCDD standard curve (t-test, two-tailed, 2 df, $p < 0.05$). As a result, multiple point estimates provided RP-bands that were valid for the

samples and suitable for risk assessment, mass balance, etc. (Fig. 2-H). The width of the RP-bands for the samples in data set I was very small (Fig. 4a). This is in agreement with the fact that the slope of the regression lines for the sample dose responses were not significantly different from that of the TCDD standard curve (t-test, two-tailed, 2 df, $p < 0.05$).

Data set II. The linear regression model described above (methods) was fit to the dose-responses (Fig. 2-A). Sample 15 showed no significant activity. Changes in the slopes of the dose-responses for samples 9, 12, and 21 suggest that the responses were approaching a maximum (Fig. 2-B; Fig. 3b), but in all cases the maximum observed was significantly greater than the efficacy of the TCDD standard $\pm$ its 95% confidence interval (Fig. 2-C,E; Fig. 3b). Samples 1 and 16 did not appear to have reached a maximal response (Fig. 2-B; Fig. 3b). They could not be tested at a greater concentration (Fig. 2-D), but their observed maximum responses were near 100%-TCDD-max (Fig. 2-F; Fig. 3b). Multiple point estimates were used to generate RP-bands for samples 1, 9, 12, 16, and 21 (Fig. 4b; Table 1). Because all the active samples showed a maximum observed response greater than 80%-TCDD-max., only $Y_i = 20\%$ -, 50% -, and 80% -TCDD-max. were considered. All the RP-bands generated were suitable for comparing among samples (Fig. 2-G,K) and no extrapolation was needed to generate the standard RP-band estimate. Unfortunately, uncertainty due to deviations from parallelism to the TCDD standard curve limited the ability to resolve and definitively rank the relative potency of the samples (Fig. 4b). The fact that the efficacy of the samples was either unknown or greater than that of the TCDD standard suggests that these estimates should not be used

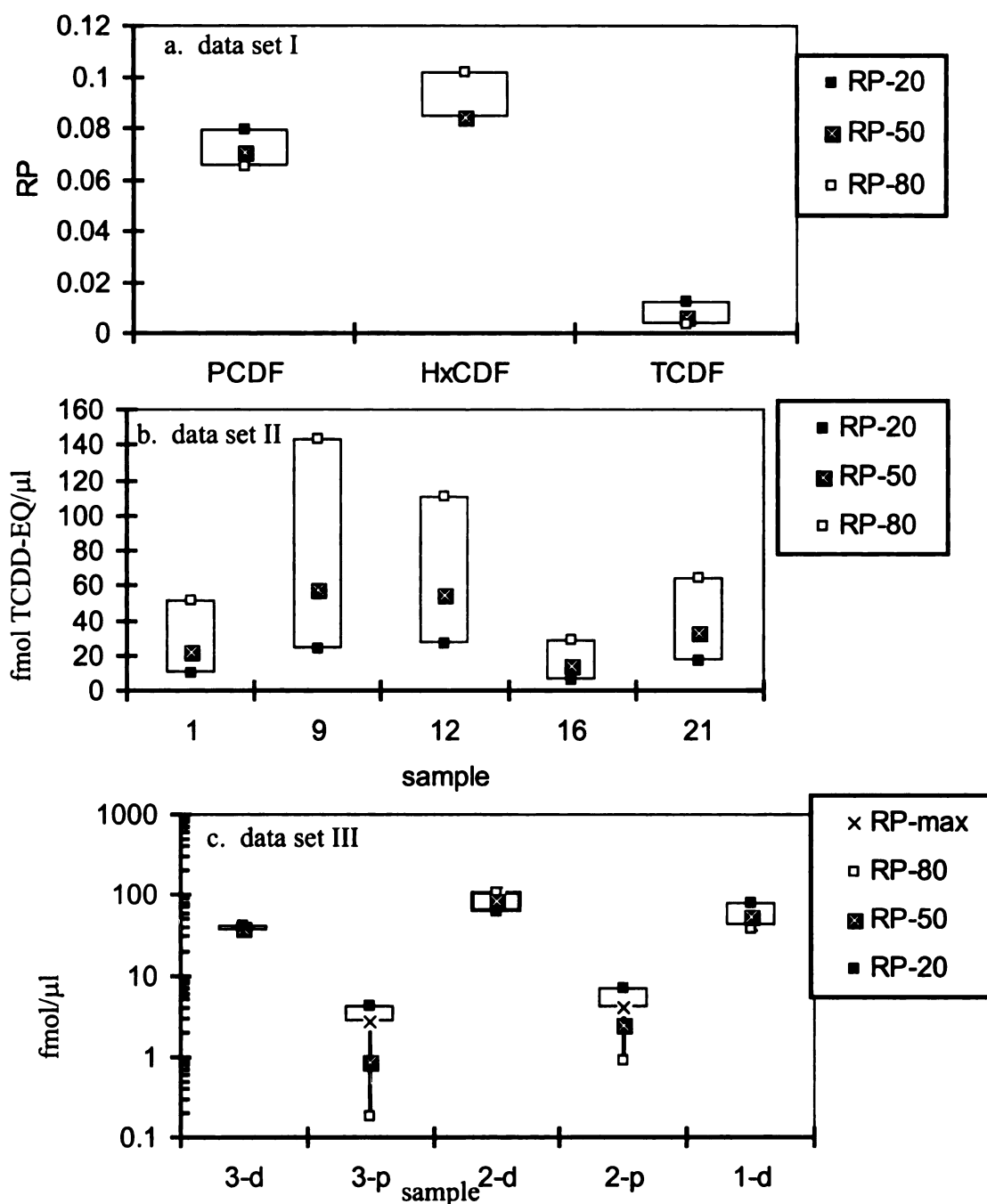


Fig. 4. Relative potency (RP) bands for three example data sets. RP-20, RP-50, and RP-80 refer to RPs calculated as a ratio of potency estimates (Equation 1) where the defined level of response (Y_i) was 20-, 50-, and 80%-TCDD-max, respectively. RP-max refers to the RP calculated at $Y_i =$ the maximum magnitude of response observed for the sample expressed as %-TCDD-max. Bars indicate regions of the RP band within the range of empirical data. A line extending beyond the bar indicates the region of the RP band which is based on extrapolation beyond the range of the empirical data.

Table 1. Relative potency estimates for example data sets (Fig. 2).

Data set-sample	RP-band ^a (RP-20 to RP-80)	RP-50 ^b	Extrapolated ^c Region	Sample ^d Efficacy
Data set I	unitless	unitless	unitless	
PCDF (n=3)	0.0445 ± 0.0303 to 0.0283 ± 0.0392	0.0353 ± 0.0323	NA	equal
HxCDF (n=3)	0.0505 ± 0.0409 to 0.143 ± 0.0493	0.0847 ± 0.00616	NA	equal
TCDF (n=3)	0.00992 ± 0.00333 to 0.00397 ± 0.00048	0.00625 ± 0.00087	NA	equal
Data set II	fmol / µl	fmol / µl	fmol / µl	
1	9.64 - 51.2	22.2	NA	unknown
9	23.2 - 143.6	57.7	NA	greater
12	27.0 - 111	54.8	NA	greater
15	NA	NA	NA	NA
16	6.32 - 28.8	13.5	NA	unknown
21	16.5 - 63.8	32.4	NA	greater
Data set III	fmol/µl	fmol/µl	fmol/µl	
3-d	41.0 - 35.0	37.9	< 35.9	less
3-p	4.16 - 0.179	0.863	< 2.70	unknown
2-d	61.6 - 107	81.2	NA	equal
2-p	7.00 - 0.872	2.47	< 3.99	unknown
1-d	77.1 - 37.8	54.0	< 41.4	less
1-p	NA	NA	NA	NA

^a RP-band = the range of relative potency estimates generated from multiple point estimates made for responses ranging from 20-80%-std.-max.

^b RP-50 = the point estimate of relative potency derived using equation 1, where 50%-std.-max. is the selected magnitude of response.

^c Refers to relative potency estimates generated at magnitudes greater than the observed efficacy for the sample. Uncertainties due to extrapolation apply.

^d Describes the observed efficacy of the sample relative to that of the standard.

NA = not applicable

in a quantitative mass balance which assumes response additivity. Use of the estimates for risk assessment purposes should consider the fact that the complex mixtures of contaminants found in these sediments may be able to elicit more efficacious responses than TCDD.

Data set III. A linear regression model was fit to the dose-responses (Fig. 3c) as described previously. A maximal response was observed for samples 1-d, 2-d, 3-d, and the TCDD standard (Fig. 2-B; Fig. 3c). In all cases, the maximal responses observed were greater than 20%-TCDD-max. (Fig. 2-C; Fig. 3c). The efficacy of samples 1-d and 3-d were markedly different from the efficacy of TCDD (Fig. 2-E; Fig. 3c). Sample 2-d had the same observed efficacy as the TCDD standard (Fig. 2-E; Fig. 3c). Sample 1-p exhibited no significant activity in the RLT 2.0 bioassay (Fig 3c). Samples 2-p and 3-p showed significant activity but did not reach a maximal response (Fig. 2-B; Fig. 3c). Due to limited sample volumes, samples 2-p and 3-p could not be tested at a greater concentrations (Fig. 2-D), but in both cases the observed maximum responses were greater than 20%-TCDD-max.

Multiple point estimates were used to calculate RP-band for samples 1-d, 2-d, 3-d, 2-p and 3-p (Fig. 4c., Table 1). The points used (Y_i) were 20-, 50-, and 80%-TCDD-max. as well as the maximum observed response for each sample if it was less than 80%-TCDD-max. The RP band generated for sample 2-d (Fig. 4c; Table 1) is valid and suitable for risk assessment (Fig. 2-H), but the uncertainty due to non-parallel slopes restricts the resolution of the estimate to somewhere between 62 and 107 fmol TCDD-EQ/ μ l (Table 1), which may or may not limit the utility of the estimate for mass balance analyses or comparing among samples. Samples 1-d and 3-d did not reach 80%-TCDD-

max., therefore some extrapolation was necessary to generate an RP-band for these samples. The extrapolated portion was minimal relative to the size of the bands, however. Thus, the RP-bands for 1-d and 3-d provide a reasonable approximation of their relative potency, but risk assessment and mass balance applications should consider the fact that they did not cause the same magnitude of response as the TCDD standard (Fig. 2-G). The efficacies of samples 2-p and 3-p were unknown and significant extrapolation was required to generate a RP-band (Fig. 4c). The broad width of the extrapolated band suggests that the extrapolated slope was quite different from that of the TCDD standard curve (Fig. 4c). As a result, the RP-band estimates for 2-p and 3-p should be applied cautiously. While they may be suitable for comparative purposes, they should not be used for risk assessment or mass balance analysis (Fig. 2-K). Based on the RP-bands calculated, the rank order of potency among the samples was $2-d \approx 1-d > 3-d > 2-p \approx 3-p > 1-p$.

Conclusions

Relative potencies based on a single ratio of point estimates are only valid when the assumptions of indirect bioassay have been met [11,14,19,20]. The example data sets illustrate that violation of these assumptions do occur, however. Sample efficacy is often either unknown or shown to be different from that of the standard. Slopes commonly vary among samples, particularly for complex mixtures of varying compositions. Overall, the situations in which the assumptions of indirect bioassay are met may be fairly rare.

The extent to which use of a single ratio of point estimates may lead to erroneous conclusions is dependent on both the degree of deviation from the assumptions of indirect bioassay and the application for which the estimate is used. In some cases, a single point estimate provides a reasonable characterization of relative potency which is suitable for the intended application. There are, however, situations in which reliance on a single point estimate can lead to erroneous conclusions. Deviations from parallelism for sample 2-p, for example, resulted in approximately 8-fold uncertainty in the relative potency estimate (Table 1). This degree of precision may be suitable for a risk assessment involving order of magnitude differences between exposure and effect concentrations and numerous safety factors. Use of a single point estimate such as the RP-50 could lead to an incorrect conclusion in a situation where the exposure and effects concentration were less than 8-fold different. Similar examples could be drawn for use of single point estimates in mass balance analyses. Thus, although a single point estimate may be suitable for certain applications even when the assumptions of indirect bioassay have been violated, they may not be suitable for all applications.

The systematic framework and relative potency estimation methods presented in this paper are not complex. They provide a simple and consistent means to evaluate the assumptions of indirect bioassay for samples of both known and unknown composition. They provide discrete relative potency estimates which can be more readily interpreted than a function. The discrete estimates, represented as a relative potency band, can be portrayed in figures and tables in such a way to make uncertainties in the estimates apparent to a reader. In this manner, it is hoped that the use of multiple point estimates

and a systematic framework for evaluating the assumptions of indirect bioassay can accommodate the need to characterize relative potency without sacrificing accuracy.

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Chapter 4

EFFECTS OF WATERBORNE EXPOSURE TO 4-NONYLPHENOL ON PLASMA SEX STEROID AND VITELLOGENIN CONCENTRATIONS IN SEXUALLY MATURE MALE CARP (*CYPRINUS CARPIO*)

D.L. Villeneuve^{*†‡}, S.A. Villalobos^{†§}, T.L. Keith^{†‡}, E.M. Snyder^{†‡}, S.D. Fitzgerald^{‡#}, J.P.
Giesy^{†‡}

[†] *Department of Zoology, † National Food Safety and Toxicology Center, ‡ Institute for
Environmental Toxicology, # Animal Health Diagnostic Lab, #Department of Pathology,
Michigan State University, East Lansing, MI, USA, 48824*

[§] *Present Address: Novartis Crop Protection, Environmental Safety, Greensboro, NC,
USA, 27419*

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Abstract

4-Nonylphenol (NP) has been shown to elicit estrogenic responses both *in vivo* and *in vitro*. The mechanism by which NP exerts estrogenic and other endocrine-modulating effects *in vivo* remains unclear, however. The goal of this study was to evaluate the ability of NP to elicit estrogenic responses through indirect mechanisms of action involving the modulation of endogenous steroid hormone concentrations. Sexually mature male common carp (*Cyprinus carpio*) were exposed to aqueous NP concentrations ranging from 0.1-10 µg NP/L (nominal) for 28-31 d. Approximately 0.5-3.5 ppm of NP was detected in pooled plasma samples or tissue samples from the carp studied. NP exposure did not significantly increase plasma concentrations of 17β-estradiol (E2), testosterone (T), or vitellogenin (VTG). Excluding outliers, plasma E2 concentrations ranged from <175 pg E2/ml to 700 pg E2/ml. T concentrations ranged from 940-24,700 pg T/ml plasma. The greatest VTG concentration detected was 52 µg/ml. One third of the plasma samples tested contained <1 µg VTG/ml. Overall, the results of this study did not support the hypothesis that exposure to waterborne NP can modulate concentrations of steroid hormones in the plasma of sexually mature male carp. The results did, however, raise a number of questions regarding the utility of estradiol equivalent (EEQ) estimates as a means of predicting *in vivo* effects of estrogenic substances. Furthermore, they provide information regarding the concentrations and variability of E2, T, and VTG in the plasma of sexually mature male carp, which may aid in design and interpretation of future studies.

1. Introduction

In recent years concern has emerged over xenobiotic chemicals which may adversely affect humans or wildlife by modulating endocrine functions through either direct or indirect mechanisms (Tyler et al. 1998, Kendall et al. 1998, Colborn et al. 1993). Alkylphenols (AP), namely nonylphenol (NP) and octylphenol (OP), have been widely regarded as suspect endocrine disrupting compounds (Nimrod and Benson 1996, Servos 1999). NP has been shown to bind to the estrogen receptor (ER) with a affinity approximately 10^{-4} to 10^{-5} times that of 17β -estradiol (E2; Lutz and Kloas 1999, Tremblay and Van Der Kraak 1998, Villeneuve et al. 1998, White et al. 1994). NP has also been shown to elicit a number of estrogenic responses *in vitro*. NP induced ER-mediated gene transcription in several recombinant cell lines (Legler et al. 1999, Villeneuve et al. 1998, Gaido et al. 1997) and significantly increased proliferation of MCF-7 human breast carcinoma cells (Soto et al. 1995). Exposure of primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and South African clawed frog (*Xenopus laevis*), to NP was found to promote vitellogenin (VTG) expression relative to controls (Smeets et al. 2000, Kloas et al. 1999, Tremblay and Van der Kraak 1998, Jobling and Sumpter 1993). Relative potencies for *in vitro* responses were also around 10^{-4} to 10^{-5} relative to E2. In addition to estrogenic responses *in vitro*, exposure to NP has also been linked to estrogenic responses *in vivo* (Kloas et al. 1999, Tremblay and Van der Kraak 1998, Christiansen et al. 1998, Madsen et al. 1997, Nimrod and Benson 1997). Thus, there is ample evidence to suggest that NP can act as an endocrine modulating compound.

The mechanism by which NP exerts estrogenic and other endocrine-disrupting effects *in vivo* remains unclear. Based on its ability to bind to the ER and induce ER-mediated reporter gene and VTG expression *in vitro*, it has been postulated that NP produces its estrogenic effects *in vivo* through a direct-acting mechanism. The proposed direct-acting mechanism involves NP binding to the ER, promoting dimerization of the ER-ligand complex, binding to genomic DNA at estrogen responsive elements (EREs), and upregulating the transcription of estrogen-responsive genes whose products are then responsible for down-stream estrogenic effects (Villeneuve et al. 1998). Recent evidence suggests that the estrogenic effects of NP may occur through indirect mechanisms. A recent study with fathead minnows (*Pimephales promelas*) linked NP exposure to increased plasma E2 concentrations in both male and females (Giesy et al. 2000). Based on a mass-balance analysis, it was hypothesized that the increased plasma E2, rather than the direct action of NP, was responsible for the increased concentrations of plasma vitellogenin which were observed (Giesy et al. 2000). An opposite effect was found in a study using Atlantic salmon (*Salmo salar*), in which exposure to NP caused a 24-43% decrease in plasma estradiol levels (Arukwe et al. 1997). Furthermore, NP exposure was linked to changes in the activities of steroid metabolizing enzymes (Arukwe et al. 1997). Other studies have associated increased ER levels with NP exposure (Nimrod and Benson 1997, White et al. 1993). Increased ER could be explained through a direct-acting effect of NP on ER gene expression, which would tend to support a direct-acting mechanism for NP. It also suggests, however, a mechanism by which NP may simply enhance the activity or effects of endogenous E2. Recent studies with daphnids have suggested that NP may also interfere with metabolic elimination of testosterone (T;

LeBlanc et al. 1999). This suggests the potential for both indirect androgenic and estrogenic effects either through the actions of elevated T or through increases in E2 facilitated by the increased concentrations of aromatizable substrate. Thus, it is unclear whether NP exerts its effects by acting directly as a xenoestrogen, or rather by promoting physiological alterations which alter either the levels or effectiveness of endogenous hormones.

Although it may seem somewhat academic, the question of mechanism is an important one. Currently, risk assessments related to the endocrine modulating effects of NP are based, primarily, on relative potencies derived for ER binding and ER-mediated responses *in vitro*. Environmental monitoring and screening of new compounds for estrogenic and other endocrine modulating activities is also at least partially based on *in vitro* responses (Ankley et al. 1998). Although *in vitro* approaches provide a measure of the direct-acting potency of NP, they cannot easily model the spectrum of potential indirect effects on endocrine homeostasis in a whole organism. The complex regulation of steroid hormone levels through the hypothalamic-pituitary-gonadal axis, via gonadotropins and both positive and negative feedback among multiple tissues has not been adequately modeled *in vitro*. Even at the level of a single tissue, few *in vitro* approaches can address responses mediated through indirect effects on the synthesis, metabolism, and/or activities of endogenous steroids. *In vitro* approaches have obvious advantages. They are generally lower in cost, shorter in duration, and avoid many of the ethical questions associated with the use of whole animals (Purchase 1999, Stokes and Marafante 1998). Thus, they can be very useful and powerful tools for research, monitoring, and risk characterization. In order for *in vitro* assays to provide viable and

accurate information, however, it is imperative to establish that the mechanism of action modeled *in vitro* is, indeed, the primary mechanism through which adverse effects may be manifested *in vivo* and/or calibrate *in vitro* responses to *in vivo* effects, particularly adverse ones.

The goal of this study was to examine some potential mechanism(s) of action for NP in fish and evaluate the results as they relate to the use and design of *in vitro* bioassays to characterize the endocrine modulating potency of NP and similar compounds. The specific objectives of the study were to 1) test the hypothesis that exposure to NP can cause significant increase in plasma E2; 2) test the hypothesis that exposure to NP can cause significant increases in plasma T; 3) calibrate *in vivo* endpoints such as plasma VTG induction and/or histopathological lesions with *in vitro* potencies reported for NP; 4) examine NP accumulation in fish tissues and plasma; and 5) characterize any indirect mechanisms suggested by the results of objectives 1 and 2.

Sexually mature, male, common carp (*Cyprinus carpio*) were chosen as the test organism for this study. Carp were commercially available, relatively easy to maintain in the laboratory, and large enough to provide tissue and plasma volumes sufficient for multiple analyses. Additionally, laboratory studies with carp would support on-going field work involving the use of VTG in feral and caged carp as a biomarker of exposure to xenoestrogens (Snyder 2000, Goodbred et al. 1997, Bevans et al. 1996, Folmar et al. 1996). Previous studies have observed both feminization and demasculinization of male carp exposed to 4-*tert*-pentylphenol (Gimeno et al. 1998a, Gimeno et al. 1998b). Finally, because previous studies with fathead minnows and Atlantic salmon provided contradictory results regarding NP's potential effect on endogenous E2 levels (Giesy et

al. 2000, Arukwe et al. 1997) it seemed prudent to examine the effect in additional species. A single gender and developmental stage was used for this study to minimize the natural variability among individual fish, allowing for greater statistical power.

2. Materials and Methods

2.1 Exposure

Sexually mature, male, common carp (*Cyprinus carpio*; 2-3 years old; 50-150 g), were obtained from J&J Aquafarms (Sanger, CA, USA). Upon arrival, 20 fish were randomly assigned to each of seven 600 L fiberglass tanks (Frigid Units, Toledo, OH, USA) and acclimated for 3 wk. Throughout acclimation and exposure, well water was delivered to each tank at a flow rate of 3.5-4 L/ min. Water temperature was maintained between 10 and 13° for the duration of the acclimation and exposure. Temperature was monitored daily using electronic Hi/Lo thermometers (Fisher Scientific, Pittsburgh, PA, USA) and electronic thermometers were calibrated weekly. Water quality parameters including ammonia, nitrite, dissolved oxygen (DO), pH, hardness, and conductivity were monitored weekly. The fish were fed daily with approximately 200 g trout chow (Silvercup, Murray, UT, USA) per tank. Waste and uneaten food were removed daily.

Fish were exposed to 4-nonylphenol (Schenectady International, Freeport, TX, USA; 95% pure) for 28-31 d using a flow through system. Concentrated solutions of NP (18.5, 5.55, 1.85, 0.555, or 0.185 mg/L) in 3.75% ethanol were delivered to treatment tanks using a variable speed peristaltic pump (MasterFlex/Cole-Parmer, Vernon Hills, IL,

USA) equipped with an 8-channel pump head (MasterFlex). Solutions were delivered at a flow rate of approximately 2 ml/min through Teflon tubing (MasterFlex). Thirty cm sections of silicon/platinum tubing (MasterFlex) were used at the pump head and were changed every 10 d due to wear. Nominal concentrations in the NP treatment tanks were 10, 3, 1, 0.3, and 0.1 µg/L. Solvent control (SC) and control tanks received 3.75% ethanol and well water, respectively, at a flow rate of approximately 2 ml per min using the same peristaltic pump and tubing. At steady state, the ethanol concentration in the treatment and SC tanks was approximately 0.002%.

NP concentrations in the exposure tanks were measured weekly. A 500 ml sample was collected from each tank using a graduated cylinder. Samples were spiked with 3.22 µg octylphenol (50 µl of 64.4 µg/ml in methanol) as an internal standard. Samples were then extracted three times (liquid/liquid extraction) with 100 ml high purity dichloromethane (DCM; Burdick and Jackson, Muskegon, MI, USA). DCM fractions were passed through 30 g anhydrous Na₂SO₄ and stored overnight. Each 300 ml DCM extract was concentrated to approximately 5 ml by rotary evaporation, and evaporated to 0.5 ml under a gentle stream nitrogen. Extracts were then transferred to high purity acetonitrile (ACN; Burdick and Jackson) by adding 1.0 ml ACN, vortexing, and evaporating again to 0.5 ml. Final extracts were analyzed by reverse phase HPLC with fluorescence detection (Snyder et al. 1999).

2.2 Sample Collection

In order to minimize variability due to diurnal fluctuations in plasma steroid concentrations (Zohar and Billard 1984) all samples were collected between the hours of 9:00 and 10:30 AM. Five fish were collected from each treatment group, each day, over the four day period from exposure day 28-31. To avoid confounding results with sampling time, individual fish were taken sequentially, from each treatment group. Fish were netted then anesthetized in 8 L of well water containing 200 mg/L MS-222 (3-aminobenzoic acid ethyl ester methane sulfonate salt; Sigma, St. Louis, MO, USA, A-5040). Immediately after opercular movement ceased, blood was drawn from the caudal vein using a 3cc disposable syringe equipped with a 21 gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) which had been rinsed with 10 mg/ml heparin (Sigma H-3393) in 0.9% NaCl. The volume of blood collected ranged from 100 μ l to 4100 μ l with a mean volume of 1700 ± 600 μ l. After collection, blood samples were immediately transferred to 15 ml disposable centrifuge tubes containing 20 μ l of 2.55 TIU/ml aprotinin (Sigma A-1153; 5.2 TIU/mg) and placed on ice. The length and weight of each fish was measured and individual fish were placed into separate plastic bags, and stored on ice until dissection.

After blood was collected from 5 fish from each treatment group, tissue samples were obtained. The gonads and hepatopancreas were removed and weighed. The brain and a gill arch were also collected, but were not weighed. A small section of each tissue was immersed in a 15 ml centrifuge tube containing 10% neutral buffered formalin and stored at room temperature. The remaining mass of each tissue (excluding gill arch) was placed in 1.0 ml cryovials and frozen immediately in liquid nitrogen. The remaining carcass was placed back in its individual plastic bag and stored at -20° C.

After collection, blood samples were stored on ice for approximately 4 hrs. Two 60 μ l samples of whole blood were taken for each fish. The remaining blood was centrifuged at 2000 rpm for 10 min at 4° C. Plasma was aliquotted into 400 μ l eppendorf tubes, 120 μ l per aliquot, and stored at -80°C along with the whole blood samples.

2.3 Estradiol Radioimmunoassay (RIA)

Plasma E2 concentrations were measured for all fish in each treatment group (except where plasma volume was insufficient). Plasma samples were extracted to separate E2 from binding proteins (McMaster et al. 1992). Two hundred μ l of plasma was combined with 800 μ l Nanopure H₂O and extracted three times with 5 ml high purity diethyl ether (Burdick and Jackson). After separation of ether and aqueous phases, samples were snap-frozen by immersion in a bath of dry ice and acetone. Ether phases from each of the three extractions were combined in a new test tube and evaporated to dryness in a 45°C water bath under a gentle stream of nitrogen. Samples were then reconstituted with 750 μ l Phosgel (McMaster et al. 1992) and stored at -80° C. Extraction efficiency, as determined by spike/recovery with tritiated estradiol (³H-E2), was $78 \pm 4\%$ (n = 4).

E2 concentrations were quantified by radioimmunoassay (RIA). The RIAs were conducted according to a protocol published by McMaster et al. (1992) with a few modifications. Tritiated estradiol (72 Ci/mmol, 3.78 μ g/ml, NEN™ Life Science, Boston, MA, USA) was diluted to 1×10^{-5} μ Ci / μ l (0.002 μ Ci per tube) in Phosgel for use in the RIA. This dilution was found to yield approximately 700 cpm per 200 μ l. Polyclonal

antibody to 17 β -estradiol (Biogenesis, Brentwood, NH, USA; AR1702) was diluted 1:20,000 for use in the RIA. This antibody dilution yielded approximately 50% binding of the added ^3H -E2 in the absence of competitor. Cross-reactivity of the antibody was reported to be 100% for 17 β -estradiol, 14% for estrone, 5% for estriol, and <0.01% for other steroid hormones (Biogenesis, AR1702, batch no. P-1). E2 standards were prepared by 2-fold serial dilution to yield 9 standards ranging from 800 to 3.125 pg. Standards, samples, non-specific binding, and total counts tubes were all run in triplicate. A total of 80 tubes were run in each assay, allowing 19 samples to be run per assay. A pooled plasma extract was run in triplicate for each assay, to provide a measure of inter-assay variation. Parallelism was tested by analyzing serial dilutions of plasma extracts (5 dilutions, n=3). Accuracy was tested by spiking extracts with known amounts of unlabelled E2 (5 concentrations, n=3).

2.4 Testosterone Enzyme Immunoassays (EIA)

Plasma T concentrations were measured for 10 fish, randomly selected from each treatment group, using enzyme immunoassay (EIA). Plasma samples were extracted to separate T from binding proteins (McMaster et al. 1992). One hundred μl of plasma was combined with 400 μl nanopure H_2O and extracted three times with 5 ml high purity diethyl ether (Burdick and Jackson). After separation of ether and aqueous phases, samples were snap-frozen by immersion in a bath of dry ice and acetone. Ether phases from each of the three extractions were combined in a new test tube and evaporated to dryness in a 45°C water bath under a gentle stream of nitrogen. Samples were then

reconstituted with 500 μ l EIA buffer (Cayman Chemical, Ann Arbor, MI, USA) and stored at -80° C. Extraction efficiency, as determined by spike/recovery with tritiated T (^{3} H-T; 96 Ci/mmol, 3.00 μ g/ml, NEN[™] Life Science, Boston, MA, USA), was $90 \pm 3\%$ (n = 5).

Testosterone EIAs were conducted in a 96 well plate format using commercial testosterone enzyme immunoassay kits (Cayman Chemical, Cat. # 582701). T standard was provided in each kit and diluted to generate T standard curves consisting of 9 concentrations ranging from 250 to 2 pg/ml. Standards were run in duplicate on each 96 well plate. Four blank and maximum binding wells were run on each plate. Non-specific binding and total activity wells were run in triplicate. Samples were run at two dilutions, 1:30 and 1:90, and each dilution was run in triplicate. Two dilutions (1:30 and 1:90) of a pooled plasma extract were run in triplicate for each assay, to provide a measure of inter-assay variation. Parallelism was tested by analyzing serial dilutions of plasma extracts (8 dilutions, n=3). Accuracy was tested by spiking extracts with known amounts of unlabelled T (4 concentrations, n=3). Cross-reactivity of the EIA antibody was reported to be 100% for testosterone, 21% for 5 α -dihydrotestosterone, 10% for 5 β -dihydrotestosterone, 3.6% for androstenedione, 1.2% for 11 β -hydroxytestosterone, and less than 0.5% for all other steroids tested (Cayman Chemical). Cross-reactivity with 11-ketotestosterone was not reported.

2.5 Vitellogenin ELISA

Plasma VTG concentrations were measured by ELISA for 12 fish randomly selected from each treatment group. The VTG ELISA protocol used and its characterization has been detailed elsewhere (Snyder 2000). The polyclonal rabbit anti-goldfish antiserum used for the VTG ELISA was developed and characterized by Nichols (Nichols 1997, Nichols et al. 1999). ELISAs were conducted using a 96 well plate format. VTG standard curves consisted of 10 serial dilutions ranging from 2730 to 5.3 ng/ml. Duplicate standard curves were run on each plate. Maximum binding and non-specific binding wells were also run in duplicate. Plasma samples (unextracted) were run at three dilutions; 1:33, 1:99, and 1:297. No significant plasma interferences were observed for samples diluted 1:25 or greater (Appendix C). Each sample dilution was run in triplicate. Accuracy, parallelism, and specificity of the VTG ELISA have been reported elsewhere (Snyder 2000).

2.6 Histological Examination

Testes, hepatopancreas, brain, and gill arch tissue from 10 fish per treatment group were examined for histological lesions. Tissues fixed in 10% neutral buffered formalin were trimmed to <0.5 cm thick sections and embedded in paraffin. Embedded tissues were sectioned at 6 μ m and stained with Hematoxylin and Eosin. Tissues were examined by a certified veterinary pathologist. All tissues were evaluated for microscopic changes including degeneration, inflammation, and neoplasia. Lesions were scored on a 0-3+ scale; 0=no lesion, 1+=mild, 2+=moderate, 3+=severe. Testes were evaluated using three different criteria; 1) active versus inactive germinal epithelium, 2)

stage of maturation, and 3) Sertoli cell proliferation. Stages of maturation were defined as follows: stage 1- thick germinal epithelium, early spermatogenesis; stage 2- moderately thick germinal epithelium, moderate spermatogenesis; stage 3- thin germinal epithelium, scattered areas of spermatogenesis. Sertoli cell proliferation was scored on a 0-3+ scale, with 0 corresponding to no proliferation and 3+ corresponding to marked proliferation.

2.7 Tissue and plasma NP concentrations

Tissue NP concentrations were quantified for four fish from the highest treatment group (10 µg/L nominal), one fish from the solvent control treatment, and one control fish. The extraction and quantification procedures used are detailed elsewhere (Snyder et al. 2000). Briefly, 20 g samples were extracted by steam distillation. NP in the extracts was quantified by normal phase HPLC with fluorescence detection.

NP concentrations in pooled plasma samples from each treatment group were also determined. Plasma from 10-15 fish from each treatment group was pooled to provide a total volume greater than 2 ml per treatment group. Two thousand µl of pooled plasma from each treatment group was spiked with 1.08 µg butylphenol (BP; 10 µl of 108 µg/ml in methanol) as an internal standard. Pooled plasma samples were then liquid/liquid extracted three times with diethyl ether. Ether layers from each extraction were pooled in a graduated test tube and evaporated to dryness at room temperature under a gentle stream of nitrogen. Samples were reconstituted in 200 µl high purity acetonitrile (Burdick and Jackson). The extraction procedure used was an adaptation of a plasma

extraction method presented by Fang et al. (2000). NP and BP concentrations in the extracts were quantified by reverse phase HPLC with fluorescence detection (Snyder et al. 1999). Nanopure water and normal goat serum (Sigma 9023) extracted and analyzed using the same procedure served as procedural blanks.

3. Results

3.1 Exposure

During the 28-31 d exposure to NP, there were few differences in exposure conditions among treatment tanks which might be expected to confound the effects of NP treatment. Water quality conditions were stable throughout the exposure period (Table 1) and no marked differences among treatment tanks were observed. Actual treatment concentrations were generally around 50% of nominal (Table 2). Less than 20% of the nominal NP concentration was detected in the 0.3 µg/L treatment tank, however (Table 2). NP concentrations were below the method detection limit (MDL) for the two lowest treatment groups as well as the control tanks (Table 2).

Although there was substantial variability in the size of the fish exposed, there were no significant differences in mean fish length or mass among treatment tanks. Mean length was 16.0 cm $\pm$ 1.5. The minimum and maximum fish lengths were 12.8 and 20.6 cm, respectively. The mean fish mass was 94.8 g $\pm$ 28.8 g, with minimum and maximum masses of 43.3 g and 207 g, respectively. Post-exposure examination of the

Table 1. Average water quality conditions during exposure.

Water Quality Parameter	Mean ^a ± Standard Deviation
Temperature ^b (°C)	11 ± 0.5
pH ^c	7.57 ± 0.03
Ammonia ^d (mg/L)	< 0.02
Nitrite ^e (mg/L)	< 0.02
Dissolved Oxygen ^f (mg/L)	10.5 ± 0.4
Hardness ^g (mg/L)	402 ± 13
Conductivity ^h (µmho)	546 ± 15

^a Averaged over all tanks and all measurements taken over the course of the exposure.

^b Monitored daily with electronic Hi/Lo thermometers (Fisher Scientific, Pittsburgh, PA, USA).

^c Monitored weekly using a Pinpoint pH meter (Aquatic Eco-systems, Apopka, FL, USA).

^d Monitored weekly using a LaMotte Ammonia Nitrogen Test Kit - low range (Aquaculture Supply, Dade City, FL, USA)

^e Monitored weekly using a LaMotte Nitrite Test Kit (Aquaculture Supply)

^f Monitored weekly using a YSI Model 57 Dissolved Oxygen Meter (YSI, Yellow Springs, OH, USA).

^g Monitored weekly using a LaMotte Hardness Test Kit (Aquaculture Supply)

^h Monitored weekly using a Pinpoint Conductivity Meter (Aquatic Eco-systems)

Table 2. Measured concentrations of 4-nonylphenol (NP) in treatment tanks.

Treatment Group (nominal NP concentration, µg NP/L)	Measured Concentration ^a (µg NP /L; mean ± st. dev.)
10	5.36 ± 0.37
3.0	1.51 ± 0.17
1.0	0.58 ± 0.07
0.3	< 0.05
0.1	< 0.05
Solvent Control	< 0.05
Control	< 0.05

^a Averaged over four separate sampling periods. Based on 500 ml samples liquid/liquid extracted with dichloromethane. Quantified by reverse phase HPLC with fluorescence detection. Concentrations were adjusted for recovery as determined by recovery of an octylphenol internal standard. Method detection limit = 0.05 µg NP/L.

carp gonads revealed that one female was exposed in the 0.3 µg/L exposure tank. The presence of a female may have confounded results from the 0.3 µg/L treatment group. All other fish exposed were male. There was one mortality during the exposure period. A fish from the 10 µg/L treatment group was observed to be swimming on its side for several days and was found lying on its side, immobile, on exposure day 16. The fish was euthanized and examined. Cranio-facial abrasions and a broken pectoral fin were detected and the gall bladder was found to be nearly black. All other fish appeared healthy throughout the exposure. No differences in gonado-somatic index (GSI) or hepatosomatic index (HSI) were detected among treatment groups. GSI ranged from 0.5-10.2%, with a mean of $3.32\% \pm 1.31\%$. HSI ranged from 0.38-2.99%, with a mean of $1.62\% \pm 0.47$. GSI and HSI were calculated relative to whole body weight after blood collection.

3.2 Plasma Estradiol

Accuracy of the plasma E2 RIA was demonstrated by analyzing plasma extracts spiked with E2 standard. Measured concentrations were plotted as a function of expected concentration (Figure 1). The slope of a regression line drawn through the points was not significantly different from 1.0 ($p < 0.749$). Dose-response curves generated by RIA analysis of several dilutions of plasma extract did not deviate markedly from parallelism to the E2 standard curve (Figure 1). The coefficient of variation (CV) among replicate

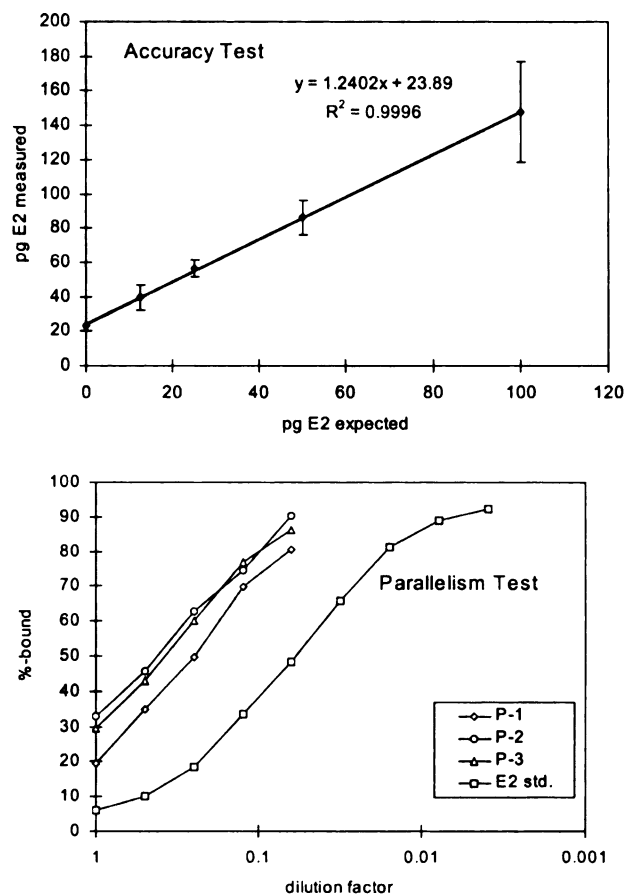


Fig. 1. Accuracy and parallelism tests for 17β -estradiol radioimmunoassay. Accuracy test was determined over the range of 20 to 80%-bound. The slope of the accuracy test plot was not significantly different from 1.0 ($p < 0.749$). The parallelism test was conducted using five serial dilutions of three separate plasma extracts (P-1, P-2, P-3). A dilution factor of 1.0 corresponds to 200 μ l of plasma extract or 800 pg of 17β -estradiol standard per sample tube.

determinations for samples was generally less than 10%, prior to correction for plasma dilution. The CV among assays was 25% (n=8) for the final calculated concentration of plasma E2.

No significant differences in plasma E2 concentrations were detected among treatment groups (Figure 2). Approximately 50% of the plasma samples contained E2 concentrations less than the MDL of 175 pg E2/ ml plasma (Figure 2). The greatest plasma E2 concentrations detected were 3370 pg E2/ml for one fish from the 10 µg/L treatment group and 2935 pg E2 / ml for the female from the 0.3 µg/L treatment. Both were considered outliers and were not included in the statistical analyses nor shown in Figure 3. Among non-outliers, the greatest plasma E2 concentration observed was 700 pg E2 /ml (Figure 2).

3.3 Plasma Testosterone

Accuracy of the T EIA was demonstrated by analyzing plasma extracts spiked with T standard. Measured concentrations were plotted as a function of expected concentration (Figure 3). The slope of a regression line drawn through the points was not significantly different from 1.0 ($p < 0.969$). EIA analysis of several dilutions of plasma extract demonstrated that extract responses were parallel to the T standard curve (Figure 3). The CV among replicate determinations for samples was generally less than 10%. The CV among plates was 9.11% (n=7) for the final calculated concentration of plasma T.

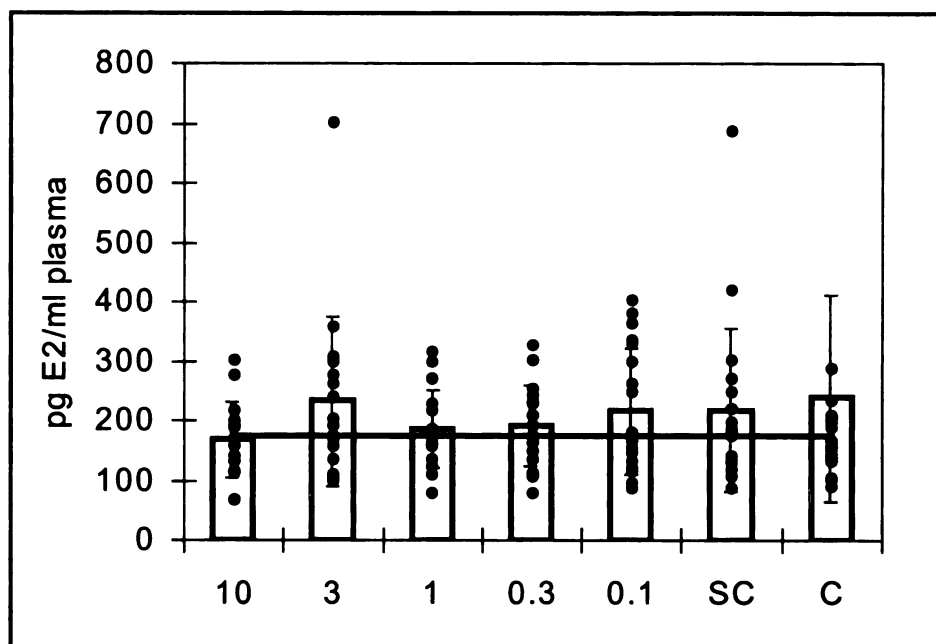


Fig. 2. Plasma estradiol (E2) concentrations, measured by radioimmunoassay, for sexually mature male carp exposed to 4-nonylphenol (NP). X-axis = nominal exposure concentrations in $\mu\text{g NP/L}$. SC = solvent control exposure. C= control exposure. Circles represent the values measured for individual fish. Columns represent the mean concentration for each treatment group. Error bars represent 1 standard deviation above and below the mean. The method detection limit (175 pg E2/ml plasma) is represented by a horizontal line.

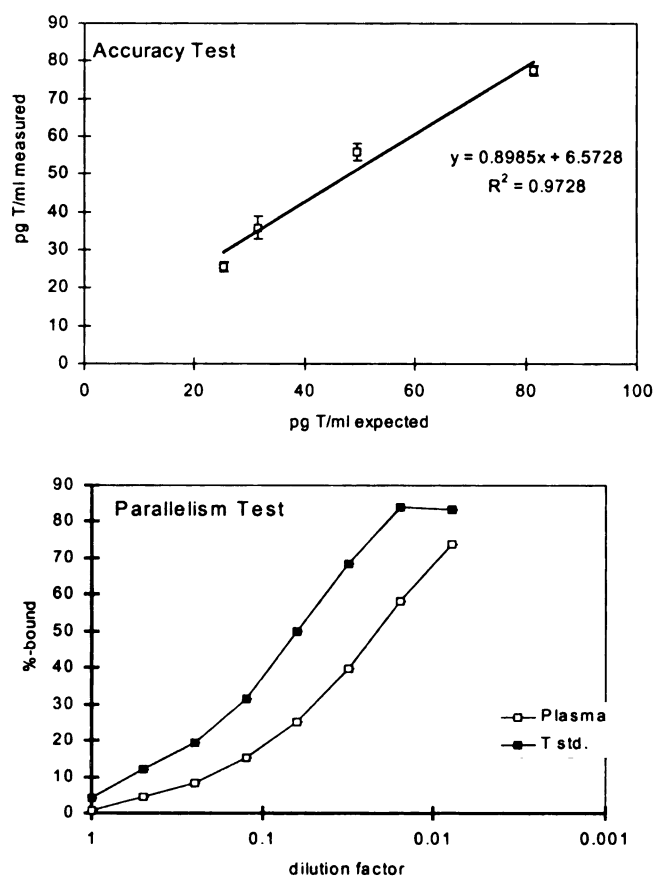


Fig. 3. Accuracy and parallelism tests for testosterone enzyme immunoassay. Accuracy was determined over the range of 20-80%-bound. The slope of the accuracy test plot was not significantly different from 1.0 ($p < 0.969$). The parallelism test was conducted using 8 serial dilutions of a pooled plasma sample ($n=3$). A dilution factor of 1.0 corresponds to 50 μ l of plasma extract or 250 pg/ml of testosterone standard per test well.

There were no significant differences in plasma T concentrations among treatments (Figure 4). All T concentrations were greater than the MDL. Plasma T concentrations ranged from 940 pg T/ml to 24.7 ng T/ml. Plasma T levels were not significantly correlated with either testes mass or GSI. There were no significant differences in E2/T ratios among treatment groups.

3.4 Plasma Vitellogenin

Accuracy and parallelism of the VTG ELISA have been demonstrated elsewhere (Snyder 2000). There were no significant differences in plasma VTG concentrations among treatment groups (Figure 5). One third of the plasma samples tested contained VTG concentrations less than the 1 µg/ml MDL (Figure 5). VTG concentrations ranged over at least two orders of magnitude (Figure 5). The greatest VTG concentration detected was 52 µg/ml. VTG concentrations were not significantly correlated with concentrations of E2 or T in the plasma, E2/T ratios, or morphological indices. The CV among replicate determinations (n=3) for samples was generally less than 10%, prior to correction for plasma dilution. The CV among plates was 44% (n=12). The pooled plasma sample used for determination of among-plate variability had a mean concentration of 2.3 ± 1.0 µg VTG/ml, which was near the MDL. Estimated concentrations VTG in the pooled plasma sample ranged from 0.8 µg/ml to 3.9 µg/ml.

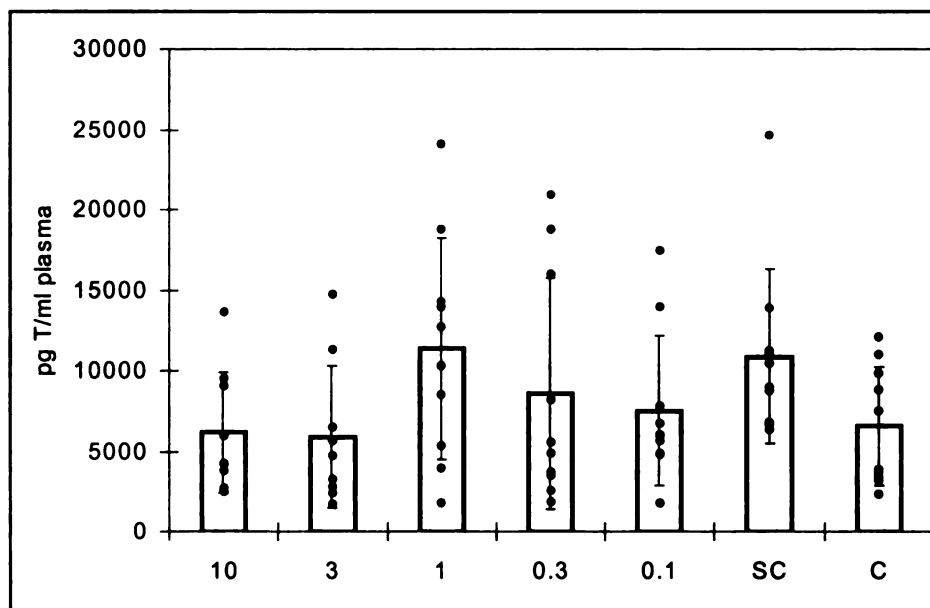


Fig. 4. Plasma testosterone (T) concentrations, measured by enzyme immunoassay, for sexually mature male carp exposed to 4-nonylphenol (NP). X-axis = nominal exposure concentrations in $\mu\text{g NP/L}$. SC = solvent control exposure. C= control exposure. Circles represent the values measured for individual fish. Columns represent the mean concentration for each treatment group. Error bars represent 1 standard deviation above and below the mean.

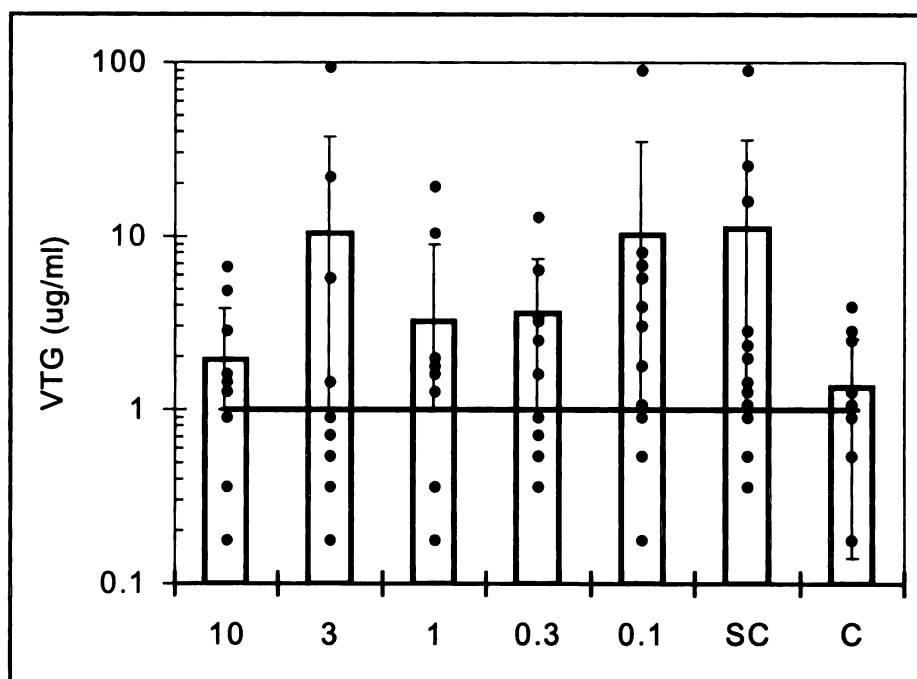


Fig. 5. Plasma vitellogenin (VTG) concentrations, measured by ELISA, for sexually mature male carp exposed to 4-nonylphenol (NP). X-axis = nominal exposure concentrations in $\mu\text{g/L}$. SC = solvent control exposure. C= control exposure. Circles represent the values measured for individual fish. Columns represent the mean concentration for each treatment group. Error bars represent 1 standard deviation above and below the mean. The method detection limit ($1.0 \mu\text{g/ml}$ plasma) is represented by a horizontal line.

3.5 Histopathology

No treatment-related lesions were observed in testes, hepatopancreas, brain, or gill. All fish examined were sexually mature. Testes were stage 2-3, with most fish being in the late spermatogenic stage. No treatment-related trends in Sertoli cell proliferation were detected. Microgranulomas, a sign of inflammation, were observed in gonad tissues from some fish, but their occurrence was not related to NP treatment. Similarly, lipidosis and/or cholestasis was observed in the hepatopancreas tissue from some fish, but again, there was no relationship between treatment group and occurrence.

3.6 Tissue and Plasma NP Concentrations

NP concentrations in tissues from four randomly selected fish exposed to a nominal concentration of 10 µg NP/L ranged from 2.1-3.8 µg NP/g tissue (Table 3). A fish randomly selected from the solvent control group contained approximately 0.016 µg NP/g tissue (Table 3). The NP concentration in the tissue of a randomly selected control fish was less than the MDL of 5 ng NP/g tissue (Table 3). NP was detected in all pooled plasma samples tested, but was not found in the procedural blanks (Table 3). Plasma pooled from fish exposed to the greatest nominal NP concentration (10 µg/L) contained 3.6 µg NP/ml plasma (Table 3). This concentration was similar to the tissue concentrations detected for that treatment group. Among the three highest treatment groups, NP concentrations in pooled plasma samples appeared to be correlated with exposure concentrations (Table 3). Among exposures of 1.0 µg/L or less, including

Table 3. Nonylphenol (NP) concentrations in tissue and pooled plasma samples.

Sample	Nominal Exposure Conc. (µg NP/L)	NP concentration ^a (µg NP/g tissue)
Tissue (a1)	10	2.33
Tissue (a4)	10	2.11
Tissue (a8)	10	3.46
Tissue (a10)	10	3.81
Tissue (c6)	Solv. Control	0.016
Tissue (d3)	Control	< 0.005
Sample	Nominal Exposure Conc. (µg NP/L)	NP concentration ^b (µg NP/ml pooled plasma)
Pooled plasma	10	3.63
Pooled plasma	3.0	1.33
Pooled plasma	1.0	0.478
Pooled plasma	0.30	NA ^c
Pooled plasma	0.10	0.452
Pooled plasma	Solv. Control	0.524
Pooled plasma	Control	0.693
Nanopure H ₂ O	Blank	< 0.0033
Normal Goat Serum	Blank	< 0.0033

^a Concentrations not adjusted for recovery. Method recoveries ≈ 80% (Snyder et al. 2000)

^b Concentrations adjusted for recovery of butylphenol internal standard. Recoveries were highly variable. Results should be considered semi-quantitative.

^c Sample lost during extraction procedure.

control groups, there was no marked difference pooled plasma NP concentrations (Table 3). Pooled plasma from the control and solvent control groups contained approximately 0.7 and 0.5 µg NP/ml, respectively (Table 3), despite the fact that water concentrations in these tanks were found to be less than 50 ng NP/L. Recoveries of the internal standard (BP) were highly variable, ranging from 80% (10 µg NP/L exposure group) to 8% (3 µg NP/L exposure group) with a median recovery of 44%. Thus, pooled plasma concentrations of NP were considered semi-quantitative.

4. Discussion

4.1 Nonylphenol exposure

The sexually mature male carp examined in this study were exposed to environmentally relevant concentrations of NP. Freshwater NP concentrations ranging from <0.010 µg/L to 180 µg/L have been reported (Bennie 1999). A study of 30 rivers in the United States reported an average NP concentration of 0.12 µg/L (Naylor et al. 1992). Actual water concentrations in this study ranged from approximately < 0.05 µg NP/L to 5 µg NP/L (Table 2). Similar studies have reported actual concentrations which were approximately 1/3 to 1/5 of the nominal concentrations (Nimrod and Benson 1998, Nichols et al. 2000). Factors such as volatilization of NP, adsorption of NP to tubing or the walls of the tanks, adsorption to organic matter present in the tanks, degradation, etc. probably account for the difference between nominal and actual exposure concentrations.

Analysis of tissue and pooled plasma extracts suggested that the carp used in this study had accumulated body burdens of NP. Concentrations as great as 3.8 $\mu\text{g NP/g}$ tissue and 3.6 $\mu\text{g NP/ml}$ pooled plasma were detected (Table 3). Based on tissue concentrations of NP detected in four randomly selected fish exposed to 10 $\mu\text{g NP/L}$ and the aqueous concentration of NP measured in the exposure tank (Table 2), the bioconcentration factor (BCF) for NP, over the course of the 28-31 d exposure period was approximately 550. BCFs reported for NP in fish range from 0.9 to 1250 (Servos 1999). BCFs determined for fathead minnows exposed for 14 and 28 d were reported to be 586 and 741, respectively (Brooke 1993). Thus, the accumulation of NP observed was similar to that reported for other studies.

The presence of NP in pooled plasma samples from the control and solvent control groups suggests either an uncontrolled exposure of the study fish to NP, or the presence of an artifact in the pooled plasma samples. NP was detected in the tissue of at least one solvent control fish. There were not sufficient resources for this study to analyze tissues from additional fish, however. There was no detectable NP in the procedural blanks for the pooled plasma extractions, suggesting that artifacts were not introduced during the sample extraction or analysis procedure. Procedural blanks were not collected or stored under the same conditions as the plasma samples, however. Thus, it is possible that NP artifacts were introduced during either the sample collection or storage phases, although there was no direct evidence to support that hypothesis. Alternatively, the fish may have been exposed to an uncontrolled source of NP, either during the laboratory exposure or during holding in the laboratory or at the commercial aquafarm. Possible sources of NP during the exposure include leaching from tubing,

laboratory plumbing, or tanks. Waterborne concentrations were below 50 ng/L, however. Assuming a BCF of 550, waterborne NP could account for up to 27.5 ppb NP in tissue or plasma. This is over an order of magnitude lower than the pooled plasma concentrations. This suggests that the most likely potential source of uncontrolled NP exposure was either the food used, or exposure prior to the laboratory study.

4.2 Morphological/Histological Endpoints

The exposure concentrations used in this study were not expected to cause significant effects on survival and/or growth. LC-50s reported for acute toxicity of NP to fish range from 125-400 µg/L (Servos 1999, Staples et al. 1998). Concentrations used in this study were at least one order of magnitude less. Furthermore, the actual water concentrations used in this study were less than NOECs reported for chronic effects of NP exposure on fish (Servos 1999). Given their stage of development and the water temperature used, the exposed carp were not expected to grow markedly during exposure. Thus, the lack of effects on morphology and survival was consistent with expectations.

Based on several reports in the literature, investigation of potential effects of NP exposure on HSI, GSI, and gonad histology seemed warranted. Injection of Atlantic salmon (*Salmo salar*) with approximately 0.1 mg NP/g caused a significant increase in HSI over a period of 10-30 d (Madsen et al. 1997). Such doses are pharmacological, however, and were nearly two orders of magnitude greater than the tissue and pooled plasma concentrations observed in this study. Currently, there are no known reports of significant changes in HSI caused by exposure to NP at environmentally relevant doses.

Exposure to 1.1 or 3.4 µg NP/L was reported to cause significant changes in numbers and size of Sertoli cells and germ cell syncytia in breeding male fathead minnows (Miles-Richardson 1999). Intraperitoneal injection of 10 or 100 µg NP/g into sexually mature male eelpout (*Zoarces viviparus*) was found to cause a significant reduction in GSI, as well as severe effects on testicular structure (Christiansen et al. 1998).

Demasculinization was observed in sexually mature male carp exposed to 4-*tert*-pentylphenol (TPP) for up to 3 months (Gimeno et al. 1998b). Similar effects were not observed for sexually mature male carp exposed as part of this study, however.

Differences among species may, at least in part, explain differences between results observed for fathead minnow or eelpout and the results of this study. Concentrations at which effects were observed in eelpout dosed with NP and carp exposed to TPP exceeded the concentrations used in this study (Christiansen et al 1998, Gimeno et al. 1998b).

Furthermore, some of the effects of TPP on GSI and testes histology were not observed until the third month of exposure (Gimeno et al. 1998b). Exposure concentrations in the fathead minnow study were similar to those used in this study, but the use of breeding pairs and a longer exposure period (42 d vs. 31 d) confounds direct comparison of study results. Thus, differences in study design could easily account for the lack of significant effects of NP exposure on GSI and gonad histology observed in this study.

4.3 Estrogenic Potency

Exposure to aqueous concentrations of NP as great as 5 µg NP/ml did not elicit significant VTG induction in sexually mature male carp (Figure 5). This is in agreement

with other studies which have demonstrated significant VTG induction only at greater exposure concentrations. Ten $\mu\text{g NP/L}$ was reported to be the threshold exposure concentration required to cause VTG induction in 2 year old rainbow trout (Jobling et al. 1996). Another study observed significant induction of VTG in rainbow trout exposed to greater than 25 $\mu\text{g NP/L}$ (Tremblay and Van der Kraak 1998). A previous study with fathead minnows observed no significant induction of VTG in males at exposure concentrations up to 2.4 $\mu\text{g NP/L}$, but significant VTG induction was observed in females (Giesy et al. 2000). Thus, compared to other reports in the literature it seems plausible that the exposure concentrations used for this study were insufficient to induce VTG in sexually mature male carp.

The lack of VTG induction, as a model estrogenic response, is a bit more surprising when one considers the estimated tissue and plasma concentrations of estradiol equivalents (EEQ) contributed by NP. The relative estrogenic potency of NP has been estimated based on estrogen response element (ERE) mediated reporter gene expression (Legler et al. 1999, Gaido et al. 1997, Villeneuve et al. 1998, White et al. 1994), VTG induction in rainbow trout hepatocytes (Tremblay and Van der Kraak 1998, White et al. 1994, Jobling and Sumpter 1993), and VTG induction *in vivo* (Tremblay and Van der Kraak 1998, Madsen et al. 1997). Relative potencies were in the range of 10^{-4} to 10^{-5} . Based on this relative potency range, and the concentrations of NP measured in tissue and pooled plasma samples, fish exposed to approximately 5 $\mu\text{g NP/L}$ were estimated to contain between 30 and 300 ng NP-derived EEQ (EEQ_{NP}) / ml plasma or g tissue. Assuming the pooled plasma concentrations (Table 3) were representative, the minimum plasma concentration of EEQ_{NP} was approximately 4.5-45 pg EEQ_{NP} /ml plasma. With

the exception of outliers, plasma E2 concentrations, ranged from < 175 pg E2/ml to 700 pg E2/ml (Figure 2). Thus, for fish from the highest exposure group, EEQ_{NP} may have exceeded endogenous EEQs by a factor of 40-400. Even in fish for the control group, NP may have contributed 1-10% of the total EEQs in the plasma. Assuming that NP functions as if it were simply a less potent form of E2, one might reasonably expect that a 40-400 fold increase in EEQ would be sufficient to yield a significant estrogenic response. For example, over the spawning cycle of female carp, plasma E2 concentrations may vary by as little as 10-fold (Aida 1988).

These observations raise a number of important questions related to the use EEQs for predicting estrogenic effects. What concentration or change in EEQs will produce a physiological effect? Are physiological responses controlled by absolute concentrations, changes in concentration, the timing or pulsatility of the changes, or combinations of all the above? What factors control the uptake and distribution of EEQs among target tissues? Unfortunately, there do not appear to be simple answers to these questions. Due to the endogenous nature of estradiol, its effects, both physiological and adverse, are intimately confounded with other variables. For example, up to 20-fold increases in E2 secretion may be associated with a temperature change as small as 5°C (Manning and Kime 1984). Positive and negative feedback controls, tissue interactions, steroid synthesis and metabolism pathways, carrier proteins, etc. all act together to modulate the activity of endogenous E2. Thus, it would be extremely difficult to develop clear dose-response (cause-effect) relationships between endogenous estradiol concentrations and estrogenic effects *in vivo*. Currently, the inability to effectively characterize such

relationships dramatically limits the utility of EEQ estimates as a basis for predicting biological effects of estrogenic substances.

4.4 Effect of NP Exposure on Plasma Steroid Concentrations

Exposure to concentrations of NP similar to those used in this study were reported to produce statistically significant increases in plasma E2 in both male and female fathead minnows (Giesy et al. 2000). In one experiment, a 10-fold increase from 2ng E2/ml to 20 ng E2/ml was observed (Giesy et al. 2000). In a second experiment, a 2-3 fold increase from 2-3 ng E2/ml to 6 ng E2/ml was detected (Giesy et al. 2000). In this study, plasma E2 concentrations varied at least 7-fold among fish (Figure 2).

Furthermore, approximately 50% of the samples had E2 concentrations which were below the MDL of 175 pg/ml. Thus, although the results of this study do not support the hypothesis that exposure to NP induced significant increases in plasma E2 in sexually mature male carp under the exposure conditions used, they do not completely reject it either. Fish to fish variability and the inability to accurately quantitate the full range of plasma E2 concentrations may have simply limited the ability to resolve the effect.

Concentrations of E2 in plasma from carp exposed in this study were markedly less than those detected in the fathead minnow study (Giesy et al. 2000). The E2 concentrations observed were very similar to those reported for feral carp, however (Goodbred et al. 1997, Folmar et al. 1996). Values reported for male carp were generally in the range of 100-800 pg/ml (Goodbred et al. 1997, Folmar et al. 1996). The mean concentration of E2 levels in serum from mature male channel catfish was reported to be

950 pg E2/ml $\pm$ 90 (Schlenk et al. 1997). Based on the results available, it was not clear whether the fact that plasma E2 concentrations in this experiment were less than plasma E2 concentrations in fathead minnows was due to species differences, differences in breeding status or developmental stage, or differences in the exposure conditions. Relative to literature values for carp, as well as those for catfish, however, the concentrations detected in this study seem reasonable.

11-ketotestosterone (11-KT) is generally regarded as the most important form of androgen for spermatogenesis in male fish (Goodbred et al. 1997). Several studies have reported a relationship between contaminant body burdens and 11-KT (Fitzsimmons 1990, Leatherland 1992). E2/11-KT or E2/T ratios have been cited as a sensitive biomarker of abnormal sex steroid concentrations (Bevans et al. 1996, Folmar et al. 1996). In at least some fish species, balance between E2 and 11-KT concentrations affects phenotypic sexual characteristics, brain and behavioral differentiation, and development of reproductive organs (Hunter and Donaldson, 1983). Male carp collected near a major metropolitan sewage treatment plant were found to have depressed plasma T concentrations (Folmar et al. 1996). Furthermore, NP was reported to interfere with metabolic elimination of T in daphnids (LeBlanc et al. 1999). Thus, there were reasons to investigate the potential for NP exposure to affect both T and 11-KT. Plasma testosterone concentrations have been shown to be closely correlated with 11-KT (Goodbred et al. 1997), and methods for measuring T were more readily available than those for 11-KT. Thus, this study focused on the effects of NP exposure on plasma T in sexually mature male carp.

Exposure to NP did not elicit significant increases in plasma testosterone in sexually mature male carp (Figure 4). Unlike for E2, all plasma samples analyzed had T concentrations which were greater than the MDL. The range of plasma T variation among fish was greater than 25-fold. This suggests that either much greater changes in concentration or much greater sample sizes would be needed to detect a significant change in plasma T resulting from NP exposure. Overall, however, the results of this study do not support the hypothesis that exposure to NP significantly altered plasma T concentrations in sexually mature male carp.

4.5 Study Limitations

Plasma sex steroids and the physiological processes they regulate can be influenced by a number of factors. Temperature is known to have dramatic effects on steroidogenesis in carp (Manning and Kime 1984). Within the optimal range for steroidogenesis, around 24-29 °C, steroid concentrations may vary by as much as 20 fold over 5°C (Manning and Kime 1984). As a result, careful control of water temperatures in the exposure tanks was critical for this study. An exposure temperature around 10°C was chosen for two reasons. First, this was a water temperature that could easily be maintained in the laboratory without the need for hot and cold water mixing which would have made it difficult to regulate flow-rates. Second, at temperatures less than 15°C there is relatively little change in plasma steroid concentrations per °C change in temperature. Thus, the study conditions were designed to minimize the effect of temperature as a confounding factor in the study. At the same time, however, it was recognized that

temperatures less than 15° C are not ideal for steroidogenesis. Thus, it was unclear whether NP could modulate steroidogenesis or steroid metabolism at the temperatures used in this study, even if it were able to do so at greater temperatures. Thus, the conclusions of this study are restricted to the range of 10-13°C temperatures maintained during the exposure.

Plasma steroid concentrations also vary with gender, stage of development, reproductive status, season, time of day, stress, etc (Goodbred 1997, Pankhurst and Dedual 1994, Down et al. 1990, Aida 1988, Pickering et al. 1987, Zohar and Billard 1984). For the purposes of this study, these factors were controlled as much as possible to reduce the variability of the endpoints and to minimize confounding factors which would obscure the potential relationship between NP exposure and changes in the endpoints measured. Single sex, sexually mature fish, were utilized to minimize variation due to differences in gender, developmental stage, or reproductive state. In order to minimize the effects of diurnal fluctuations, sample collection was restricted to the same 1.5 hr period on each sampling day. Thus, these factors should not have markedly affected study results. For comparative purposes, however, results of this study will only be directly comparable to other studies using sexually mature male carp exposed at 10-13° C for 28-31 d at approximately the same time of year. Numerous studies using a variety of species and conditions are needed to fully elucidate the potential mechanisms by which NP may elicit endocrine disrupting effects on fish.

4.6 Conclusions

Under the conditions used for this study, exposure to NP did not elicit a statistically significant increase in plasma E2, plasma T, or plasma VTG concentrations. Furthermore, no-treatment related changes in gonad histology or morphological parameters were observed. The results of this study do not provide support for the hypothesis that NP can elicit endocrine modulating effects in fish through an indirect mechanism of action. They do not, however, provide evidence to reject that hypothesis. Exposure to NP was confirmed by the detection of approximately 0.5-3.5 ppm of NP in pooled plasma or tissue samples from the carp studied. The lack of significant VTG induction, despite the fact that NP may have contributed between 30 and 300 ng EEQ_{NP} / ml plasma or g fish tissue, raises a number of questions regarding the utility of EEQ estimates for predicting biological responses. Additionally, because of the lack of a significant estrogenic response, it was not possible to calibrate the *in vivo* potency of NP for producing an estrogenic effect in sexually mature male carp with *in vitro* potencies reported for NP. The study does, however, provide useful information regarding the concentrations and variability of plasma steroids and plasma VTG in sexually mature male carp, which may aid in the design or interpretation of future studies.

5. Acknowledgements

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SUMMARY AND CONCLUSIONS

The studies described in this dissertation centered around the application of mechanism-specific *in vitro* bioassays in the field of environmental toxicology. The first study (Chapter 1) examined interactions between environmental xenobiotics and estrogen receptor-mediated responses. Two types of *in vitro* assays were used to screen and rank the potential estrogenicity of 11 chemicals based on their affinity to bind to the estrogen receptor (ER) and/or induce estrogen response element (ERE)-mediated reporter gene expression in MCF-7-luc cells. Six of the 11 compounds tested were able to displace tritiated 17 β -estradiol from the ER. Their rank order of affinity was 17 β -estradiol (E2) > coumestrol > 17 β -ethynyl-estradiol (EE2) > nonylphenol (NP) > octylphenol (OP) > bisphenol A (BPA). Indole-3-carbinol, atrazine, *o,p'*-DDE, *p,p'*-DDE, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) had no measurable affinity for the ER. The ability of each compound to induce estrogen response element (ERE)-mediated gene transcription was measured using the MCF-7-luc *in vitro* bioassay. The rank order of potency for producing luciferase expression in the MCF-7-luc bioassay was E2 > EE2 > NP > OP > coumestrol > atrazine > BPA > indole-3-carbinol. TCDD, *o,p'*-DDE, and *p,p'*-DDE did not induce luciferase expression. TCDD was shown to antagonize the effect of E2 in the MCF-7-luc bioassay. In addition to screening and ranking, the assay-specific potencies reported in Chapter 1 were later applied in several studies involving the use of mass-balance analysis to aid in the identification of compounds or classes of compounds responsible for the estrogenicity of various environmental samples (Appendix A). Finally, comparison of receptor binding affinity and gene expression

assay results was used to generate several hypotheses related to the mechanism of action of the compounds tested. Relative binding affinity for calf ER was correlated with the potency for gene expression in MCF-7-luc cells, but was not predictive of efficacy. The “ligand insertion hypothesis” was cited as one potential explanation for the lack of correlation between receptor binding affinity and efficacy. It was also noted that atrazine, which showed no affinity for the ER, was able to induce ERE mediated gene expression, suggesting a non-ER-mediated mechanism for the *in vitro* estrogenic effects of atrazine. Thus, Chapter 1 provided a useful overview of potential uses for *in vitro* bioassays in environmental toxicology research.

The second study presented in this dissertation (Chapter 2) focused on the development and characterization of a recombinant, rainbow trout cell line-based, assay for assessing dioxin-like potency. The RLT 2.0 bioassay developed by Richter et al. (1997) was adapted to a 96-well plate format to increase assay efficiency. RLT 2.0-specific relative potencies (REPs) were derived for a number of halogenated aromatic hydrocarbons (HAHs) and compared to REPs based on other fish and mammalian bioassays, both *in vitro* and *in vivo*. Overall, the REPs and rank ordering of HAHs based on the RLT 2.0 assay was similar to those based on other rainbow trout-specific bioassays *in vitro* and *in vivo*. This helped establish the utility of the RLT 2.0 bioassay for evaluating dioxin-like potency of HAHs to fish. Because rank ordering and REPs were relatively similar to those for mammalian assays, however, it was not clear whether the fish cell line-based RLT 2.0 assay is more relevant for predicting effects in fish than analogous mammalian cell-based assays. Sensitivity analysis indicated that variability in RLT 2.0-based REP estimates yields approximately 10-fold uncertainty in TCDD

equivalents (TEQ) estimates calculated using RLT 2.0-derived values. This uncertainty estimate was important for future application of the RLT 2.0 assay in studies involving mass-balance analysis. Differences of less than one order of magnitude between instrumentally derived TEQ and RLT 2.0 bioassay-derived TCDD equivalents (TCDD-EQ) may not be relevant. Thus, Chapter 2 illustrates the type of method development and characterization needed to establish and enhance the utility of a mechanism-specific *in vitro* bioassays as tools for environmental toxicology research and risk characterization.

The study described in Chapter 3 focused on the current debate surrounding the derivation, use, and misuse of *in vitro* bioassay-based REP estimates in risk assessment and environmental toxicology research. Chapter 3 presented a systematic approach which was developed for evaluating the assumptions underlying REP estimation. Furthermore, it described the use of multiple point estimates and relative potency bands to characterize REPs in situations where parallelism between sample and standard dose-response relationships cannot be demonstrated. The methods described were demonstrated using three example data sets and have been subsequently applied in a number of other studies in our laboratory (Appendix A)

The fourth study described (Chapter 4) was an *in vivo* study designed, in part, to address the relevance of current *in vitro* models for predicting estrogenic potencies in fish, *in vivo*. Previous studies suggested that 4-nonylphenol (NP) may elicit estrogenic effects in fish *in vivo* by modulating plasma steroid hormone concentrations. The study described in Chapter 4 detected no significant increases in concentrations of E2, testosterone (T), or vitellogenin (VTG) in plasma from sexually mature male common

carp exposed to waterborne NP for 28-31 d at 10-13°C. Thus, the study did not provide evidence to support the hypothesis that NP may mediate estrogenic effects through an indirect mechanism involving elevation of steroid hormone concentrations in plasma. The lack of a detectable estrogenic effect *in vivo* as a result of exposure to NP, hindered the ability to calibrate the known *in vitro* potency of NP to its potency for producing estrogenic effects in sexually mature male carp. The lack of estrogenic response also raised a number of questions regarding the utility of estimating plasma or tissue concentrations of 17 β -estradiol equivalents (EEQ) as a means of predicting the potential for estrogenic effects *in vivo*.

There is clearly a need and demand to develop and establish the utility of mechanism-specific *in vitro* bioassays as tool in environmental toxicology research and risk characterization. The studies presented in this dissertation exemplify the type of research that is needed to meet this demand. To a large extent, the technology and understanding of molecular and cellular processes needed to develop effective *in vitro* tools is already in place. The array of *in vitro* assays developed to address the emerging issue of environmental estrogens (Chapter 1) provides evidence for this. What has lagged behind, however, is research aimed at correlating and calibrating *in vitro* responses to effects *in vivo*. The paucity of effective studies correlating responses *in vitro* with effects *in vivo* is currently the greatest hindrance to the widespread use and utility of *in vitro* bioassays as risk assessment tools. The utility of *in vitro* bioassay results could also be increased through the development of standardized, widely accepted, and reliable methods for analyzing and interpreting *in vitro* bioassay results.

Given such developments, however, mechanism-specific *in vitro* bioassays can be important and powerful tools for environmental toxicology research and risk assessment.

Reference:

Richter CA, Tieber VL, Denison MS, Giesy JP. 1997. An *in vitro* rainbow trout cell bioassay for aryl hydrocarbon receptor-mediated toxins. *Environ Toxicol Chem.* 16:543-550.

APPENDIX A

RELATED PUBLICATIONS

Villeneuve, D.L., J.S. Khim, K. Kannan, J.P. Giesy. 2000. Response of fish and mammalian *in vitro* bioassays to complex mixtures of polychlorinated naphthalenes, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. (Submitted to *Aquat. Toxicol.* 1/5/00)

Villeneuve, D.L., J.S. Khim, K. Kannan, J. Falandysz, A.L. Blankenship, J.P. Giesy. 2000. Relative potencies of individual polychlorinated naphthalenes to induce dioxin-like responses in fish and mammalian *in vitro* bioassays. (Submitted to *Arch. Environ. Contam. Toxicol.* 12/1/99)

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Snyder, S.A., D.L. Villeneuve, E.M. Snyder, J.P. Giesy. 2000. Toxicity identification and evaluation of estrogenic and dioxin-like compounds in wastewater effluents. (Submitted to *Environ. Sci. Technol.* 2/00).

Kannan, K., D.L. Villeneuve, N. Yamashita, T. Imagawa, S. Hashimoto, A. Miyazaki, J.P. Giesy. 2000. Vertical profiles of dioxin-like and estrogenic activities associated with a sediment core from Tokyo Bay, Japan. (Submitted to *Environ. Sci. Technol.* 2/21/00).

Yamashita, N., K. Kannan, T. Imagawa, D.L. Villeneuve, S. Hashimoto, A. Miyazaki, J.P. Giesy. 2000. Vertical profile of polychlorinated dibenzo-*p*-dioxins, -dibenzofurans, -naphthalenes, -biphenyls, polycyclic aromatic hydrocarbons, and alkylphenols in a sediment core from Tokyo Bay, Japan. (Submitted to *Environ. Sci. Technol.* 2/21/00)

Khim, J.S., D.L. Villeneuve, K. Kannan, W.Y. Hu, J.P. Giesy, S.G. Kang, K.J. Song, C.H. Koh. 2000. Instrumental and bioanalytical measures of persistent organochlorines in blue mussel (*Mytilus edulis*) from Korean coastal waters. (Submitted to *Arch. Environ. Contam. Toxicol.* 11/2/99).

Giesy, J.P., S.L. Pierens, E.M. Snyder, S. Miles-Richardson, V.J. Kramer, S.A. Snyder, K.M. Nichols, and D.L. Villeneuve. 2000. Effects of 4-nonyl phenol on

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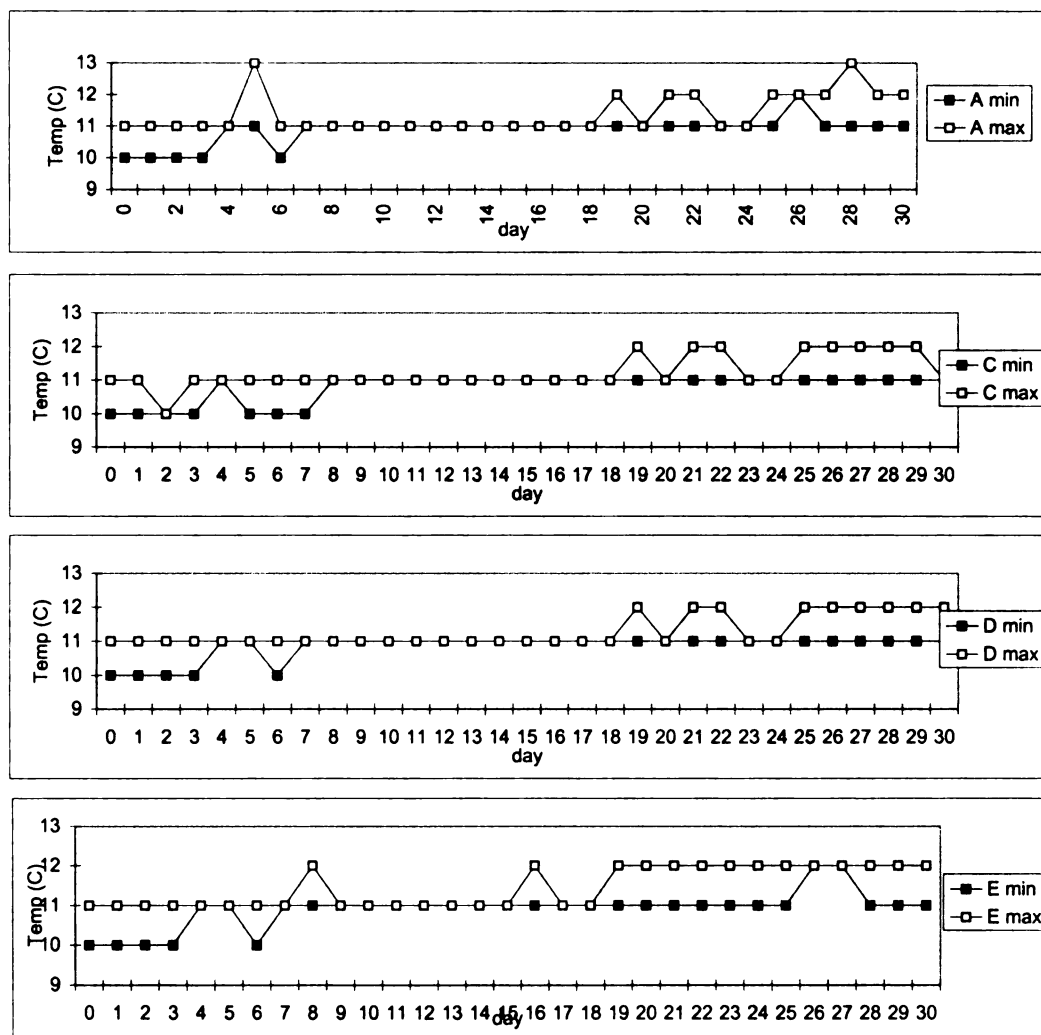
Kannan K., N. Yamashita, D.L. Villeneuve, S. Hashimoto, A. Miyazaki, and J.P. Giesy. 1999. Vertical profile of dioxin-like and estrogenic potencies in a sediment core from Tokyo Bay, Japan. *Organohalogen Compounds* 42:33-38 (short paper).

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APPENDIX B

RAW DATA FROM WATERBORNE EXPOSURE OF SEXUALLY MATURE MALE COMMON CARP (*CYPRINUS CARPIO*) TO 4-NONYLPHENOL

Fig. B.1. Temperature profiles over the course of the exposure of sexually mature male carp to 4-nonylphenol (NP).

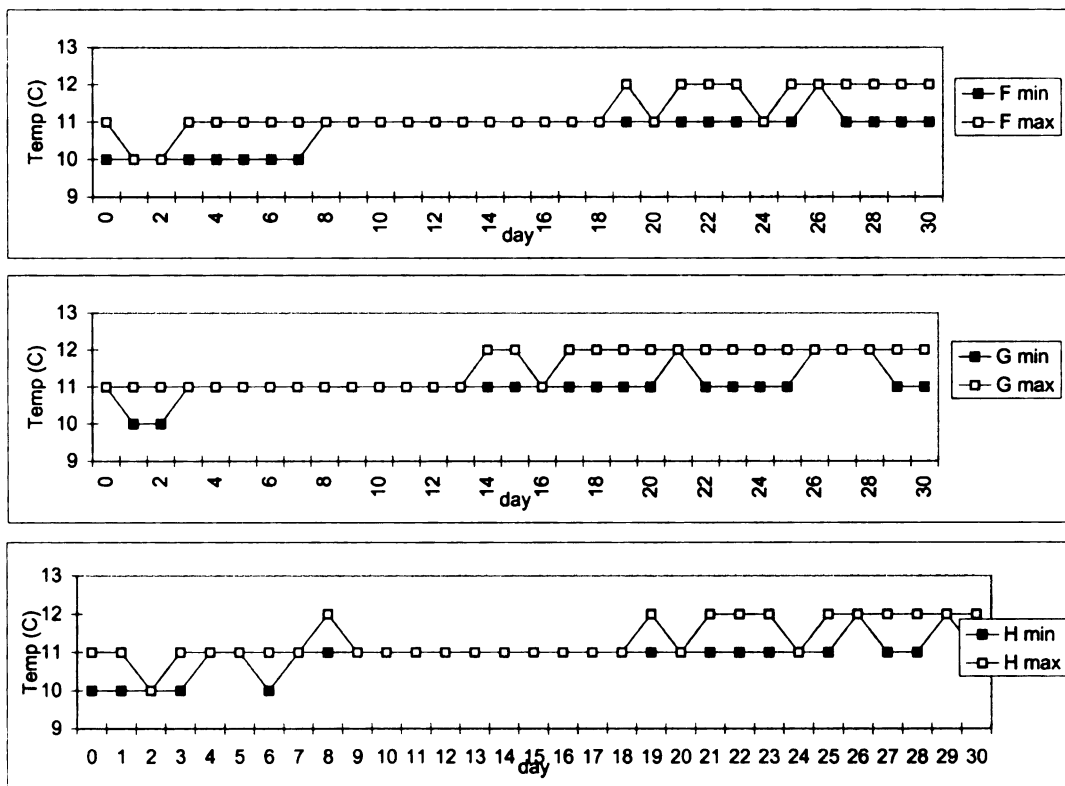


min = minimum temperature max = maximum temperature

Temperatures monitored daily with electronic Hi/Lo thermometers (Fischer Scientific, Pittsburgh, PA, USA).

A = 10 ug/L; C = solvent control; D = control; E = 1 ug/L; F = 0.3 ug/L; G = 0.1 ug/L; H = 3.0 ug/L; nominal NP concentration in tank

Fig. B.1. (continued)



min = minimum temperature max = maximum temperature

Temperatures monitored daily with electronic Hi/Lo thermometers (Fischer Scientific, Pittsburgh, PA, USA).

A = 10 ug/L; C = solvent control; D = control; E = 1 ug/L; F = 0.3 ug/L; G = 0.1 ug/L H = 3.0 ug/L; nominal NP concentration in tank

Table B.1. Results of weekly water quality monitoring over the course of exposure of sexually mature male carp to 4-nonylphenol (NP).

Date	Tank	pH	NH-3 mg/L	NO-2 mg/L	DO mg/L	Hardness mg/L	Conduct. umho
3/16/99	A	7.57	<0.02	<0.02	10	400	546
	B	7.58	<0.02	<0.02	10	390	537
	C	7.57	<0.02	<0.02	9.8	390	542
	D	7.58	<0.02	<0.02	9.8	380	546
	E	7.56	<0.02	<0.02	9.8	380	546
	F	7.56	<0.02	<0.02	9.8	390	543
	G	7.57	<0.02	<0.02	9.8	390	546
	H	7.57	<0.02	<0.02	9.8	390	539
3/23/99	A	7.6	<0.02	<0.02	10.6	400	533
	B	7.61	<0.02	<0.02	10.7	400	530
	C	7.6	<0.02	<0.02	10.8	400	531
	D	7.6	<0.02	<0.02	10.8	400	527
	E	7.59	<0.02	<0.02	10.8	410	509
	F	7.59	<0.02	<0.02	10.8	408	531
	G	7.6	<0.02	<0.02	10.8	420	563
	H	7.59	<0.02	<0.02	10.8	400	557
3/30/99	A	7.58	<0.02	<0.02	10.9	400	577
	B	7.65	<0.02	<0.02	10.9	425	567
	C	7.58	<0.02	<0.02	10.9	410	567
	D	7.59	<0.02	<0.02	10.8	410	565
	E	7.57	<0.02	<0.02	10.7	425	567
	F	7.58	<0.02	<0.02	10.7	425	559
	G	7.58	<0.02	<0.02	10.7	410	564
	H	7.57	<0.02	<0.02	10.4	410	557
4/6/99	A	7.53	<0.02	<0.02	10.4	400	544
	B	7.53	<0.02	<0.02	10.6	400	537
	C	7.51	<0.02	<0.02	10.6	nm	538
	D	7.55	<0.02	<0.02	10.6	nm	540
	E	7.51	<0.02	<0.02	10.6	nm	543
	F	7.52	<0.02	<0.02	10.6	nm	539
	G	7.52	<0.02	<0.02	10.6	nm	544
	H	7.5	<0.02	<0.02	10.3	nm	539
Mean		7.57	---	---	10.5	402	546
SD		0.03	---	---	0.4	12.6	14.7
CV		0.44	---	---	3.8	3.1	2.7

A = 10 ug/L; C = solvent control; D = control; E = 1 ug/L; F = 0.3 ug/L;

G = 0.1 ug/L H = 3.0 ug/L; nominal NP concentration in tank

Tank B was not part of this study.

pH measured with a Pinpoint pH meter (Aquatic Eco-systems, Apopka, FL, USA)

NH-3 = Ammonia; measured with a LaMotte Ammonia Nitrogen Test Kit - low range
(Aquaculture Supply, Dade City, FL, USA)

NO-2 = Nitrite; measured with a LaMotte Nitrite Test Kit (Aquaculture Supply)

DO = Dissolved oxygen; measured with a YSI Model 57 Dissolved Oxygen Meter
(YSI, Yellow Springs, OH, USA)

Hardness measured with a LaMotte Hardness Test Kit (Aquaculture Supply)

nm = no measurement; ran out of kit reagents

Conduct. = Conductivity; measured with a Pinpoint Conductivity Meter (Aquatic Eco-systems)

Table B.2. Blood volume collected, length, mass, gonad mass, hepatopancreas mass, gonado-somatic index (GSI), hepato-somatic index (HSI), and duration of exposure (time d) for individual sexually mature male carp exposed to 4-nonylphenol at the nominal concentrations indicated below.

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	hp (g)	GSI (%)	HSI (%)
D1	27	control	800	13.7	53.5	1.757	0.21057	3.28	0.39
d2	27	control	1800	14.6	80.9	0.63531	1.30662	0.79	1.62
d3	27	control	2400	17.6	121.8	4.385	2.1346	3.60	1.75
d4	27	control	2300	17.2	125.6	4.69882	1.78915	3.74	1.42
d5	27	control	2200	15	83.5	2.8637	1.47	3.43	1.76
d6	28	control	2400	17.5	135.7	4.214	2.8133	3.11	2.07
d7	28	control	1100	15.8	90.9	3.0777	1.5763	3.39	1.73
d8	28	control	1400	15.3	74.7	1.9734	1.5849	2.64	2.12
d9	28	control	2000	17.6	128.5	3.7452	3.3926	2.91	2.64
d10	28	control	1800	16.1	93.4	2.9781	1.6256	3.19	1.74
d11	29	control	2200	16.7	103.5	2.253	1.1624	2.18	1.12
d12	29	control	1500	16.1	81.2	3.9125	1.2384	4.82	1.53
d13	29	control	3000	18.1	125.6	3.4927	1.7862	2.78	1.42
d14	29	control	800	15	73.7	2.6472	1.5177	3.59	2.06
d15	29	control	1200	15.3	91.5	1.9245	1.7097	2.10	1.87
d16	30	control	1500	13.5	55	1.3849	0.8402	2.52	1.53
d17	30	control	1800	16.5	101.9	3.79803	1.576	3.73	1.55
d18	30	control	1200	14.9	74.5	3.07143	1.53093	4.12	2.05
d19	30	control	1800	14	66.9	1.4034	1.467	2.10	2.19
d20	30	control	2200	15.6	90.5	2.3891	1.68671	2.64	1.86
Mean			1770	15.8	92.6	2.83025	1.62094	3.03	1.72
SD			583	1.36	24.4	1.11356	0.64957	0.87	0.46
CV			32.9	8.60	26.4	39.3	40.1	28.85	26.77

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	hp (g)	GSI (%)	HSI (%)
c1	27	S.C.	2000	18.5	150.4	3.97341	4.22042	2.64	2.81
c2	27	S.C.	800	14.3	68.4	2.7392	0.762237	4.00	1.11
c3	27	S.C.	1700	17	114.5	4.4003	1.74	3.84	1.52
c4	27	S.C.	2300	16.5	112.2	5.08457	0.82609	4.53	0.74
c5	27	S.C.	1000	14	62.4	2.3753	0.90514	3.81	1.45
c6	28	S.C.	2000	16	97	2.2854	2.0756	2.36	2.14
c7	28	S.C.	1000	15.3	79.4	1.1448	0.9985	1.44	1.26
c8	28	S.C.	1400	15	53.5	1.9069	1.3563	3.56	2.54
c9	28	S.C.	2300	17.1	112.7	5.1165	1.6246	4.54	1.44
c10	28	S.C.	1600	14.5	65.4	2.1758	1.0003	3.33	1.53
c11	29	S.C.	1100	14.1	61.2	4.4283	0.3861	7.24	0.63
c12	29	S.C.	3200	19.2	172.4	6.7389	2.0452	3.91	1.19
c13	29	S.C.	1700	14.6	77.7	1.8398	1.3633	2.37	1.75
c14	29	S.C.	2300	16.8	104.9	2.5843	1.963	2.46	1.87
c15	29	S.C.	2900	17.5	123.6	3.1813	2.176	2.57	1.76
c16	30	S.C.	1700	15	81.1	3.95022	0.8852	4.87	1.09
c17	30	S.C.	1900	16	87.9	3.91827	1.37	4.46	1.56
c18	30	S.C.	700	13	52	1.90752	0.94295	3.67	1.81
c19	30	S.C.	2500	15.9	95.8	2.6012	1.31301	2.72	1.37
c20	30	S.C.	2100	15.5	81	1.07739	1.29003	1.33	1.59
c21	30	S.C.	1700	15.1	74.8	3.02034	0.83616	4.04	1.12
Mean			1805	15.8	91.8	3.16427	1.43239	3.51	1.54
SD			664	1.55	31.2	1.44600	0.80735	1.32	0.53
CV			36.8	9.82	34.0	45.7	56.4	37.5	34.2

Table B.2. (continued)

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	hp (g)	GSI (%)	HSI (%)
g1	27	0.1	2400	17	110.7	3.2312	1.93503	2.92	1.75
g2	27	0.1	1500	17.9	133.6	3.48636	3.07345	2.61	2.30
g3	27	0.1	1800	16.9	106.1	2.2535	1.6724	2.12	1.58
g4	27	0.1	800	15.7	96.5	3.22408	1.1198	3.34	1.16
g5	27	0.1	2200	17.3	110.1	2.91226	2.19941	2.65	2.00
g6	28	0.1	1600	14.5	165.8	1.5246	0.6309	0.92	0.38
g7	28	0.1	2000	16.7	101.3	4.6432	1.7809	4.58	1.76
g8	28	0.1	1500	15.4	76	3.2346	1.0339	4.26	1.36
g9	28	0.1	2200	17.3	118.2	4.0266	1.506	3.41	1.27
g10	28	0.1	1000	15.8	91.2	2.9243	1.3885	3.21	1.52
g11	29	0.1	1300	16.3	93.1	3.8056	1.5088	4.09	1.62
g12	29	0.1	1700	15	70.3	2.9256	0.9968	4.16	1.42
g13	29	0.1	1700	14.5	64	1.369	1.3714	2.14	2.14
g14	29	0.1	1800	15.7	78.4	1.0325	0.8981	1.32	1.15
g15	29	0.1	1600	16	95.6	3.6046	1.8872	3.77	1.97
g16	30	0.1	2000	15.8	89.1	3.1352	1.2258	3.52	1.38
g17	30	0.1	1800	17.1	104.8	3.3001	1.85825	3.15	1.77
g18	30	0.1	2000	14.6	72.2	1.75212	0.56474	2.43	0.78
g19	30	0.1	1600	14.1	70	2.1501	0.72502	3.07	1.04
Mean			1711	16.0	97.2	2.87029	1.44086	3.03	1.49
SD			398	1.11	24.8	0.95790	0.61404	0.97	0.48
CV			23.3	6.96	25.5	33.4	42.6	32.1	31.9

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	hp (g)	GSI (%)	HSI (%)
f1	27	0.3	1200	17.7	140.6	6.89015	4.20506	4.90	2.99
f2	27	0.3	2200	16.1	103.4	5.09631	2.35151	4.93	2.27
f3	27	0.3	1700	17.5	112.5	4.37504	1.79755	3.89	1.60
f4	27	0.3	3000	18.5	143.6	5.43126	2.88449	3.78	2.01
f5	27	0.3	2000	15.5	90.8	4.1596	1.71012	4.58	1.88
f6	28	0.3	2800	18	131.2	5.7918	1.5984	4.41	1.22
f7	28	0.3	1800	18	130.8	5.3373	2.1175	4.08	1.62
f8	28	0.3	2100	16.2	82.1	2.2708	0.8699	2.77	1.06
f9	28	0.3	1100	14	66.9	2.7776	1.1525	4.15	1.72
f10	28	0.3	1500	16	90.7	2.632	1.1645	2.90	1.28
f11	29	0.3	1600	15.1	77.4	3.4591	0.9156	4.47	1.18
f12	29	0.3	1700	15.7	94.8	9.6727	2.1954	10.20	2.32
f13	29	0.3	1700	15.2	79.1	0.9295	1.5803	1.18	2.00
f14	29	0.3	1700	15.5	78.5	3.6662	1.5357	4.67	1.96
f15	29	0.3	1700	16.2	94.2	1.1289	2.0749	1.20	2.20
f16	30	0.3	1800	15.7	88.9	4.17535	0.65057	4.70	0.73
f17	30	0.3	1800	16.5	99.5	2.954	1.43282	2.97	1.44
f18	30	0.3	2000	16.6	98	2.75294	1.02731	2.81	1.05
f19	30	0.3	500	14.1	61	1.5541	1.21414	2.55	1.99
Mean			1784	16.2	98.1	3.95024	1.70938	3.95	1.71
SD			552	1.27	23.9	2.13430	0.83249	1.90	0.55
CV			30.9	7.82	24.4	54.0	48.7	48.1	32.4

Table B.2. (continued)

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	hp (g)	GSI (%)	HSI (%)
e1	27	1	1400	15	82.1	3.79961	1.47332	4.63	1.79
e2	27	1	400	18.3	137.4	3.85092	2.65732	2.80	1.93
e3	27	1	1800	15.1	80.9	4.24401	1.43	5.25	1.77
e4	27	1	2900	18.6	143	4.47401	2.50955	3.13	1.75
e5	27	1	3000	18.6	151.4	3.14216	3.21218	2.08	2.12
e6	28	1	1100	14.5	62.4	0.776	0.9395	1.24	1.51
e7	28	1	1600	14.4	56.8	0.521	1.032	0.92	1.82
e8	28	1	1300	14.7	43.3	1.6992	0.9944	3.92	2.30
e9	28	1	4100	20.6	206.9	7.9936	5.3495	3.86	2.59
e10	28	1	1500	17.7	120.2	4.1787	1.0992	3.48	0.91
e11	29	1	100	12.8	48.7	2.0337	0.8294	4.18	1.70
e12	29	1	1600	14.6	70.5	2.6908	0.9482	3.82	1.34
e13	29	1	1700	15.7	89.7	2.9794	1.1068	3.32	1.23
e14	29	1	2900	16.3	99.3	3.382	2.29	3.41	2.31
e15	29	1	1800	15.8	83.7	2.7934	1.294	3.34	1.55
e16	30	1	2100	17	111.3	2.08	1.7265	1.87	1.55
e17	30	1	1300	16.7	108	4.0576	1.905	3.76	1.76
e18	30	1	2400	17.6	130.7	7.53862	1.7458	5.77	1.34
e19	30	1	2600	18.5	136.1	4.25165	2.3104	3.12	1.70
e20	30	1	3000	17.9	134.8	4.67539	2.6518	3.47	1.97
Mean			1930	16.5	104.9	3.55809	1.87524	3.37	1.75
SD			960	1.95	41.0	1.86575	1.07806	1.20	0.40
CV			49.8	11.8	39.1	52.4	57.5	35.6	22.7

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	hp (g)	GSI (%)	HSI (%)
h1	27	3	1700	18.4	126.8	5.62726	1.86302	4.44	1.47
h2	27	3	2000	18.5	132.6	5.14829	2.37267	3.88	1.79
h3	27	3	3000	18.1	135.4	1.31	2.25401	0.97	1.66
h4	27	3	800	15.5	83.1	2.701	1.13207	3.25	1.36
h5	27	3	500	16.3	97.8	2.90587	1.939	2.97	1.98
h6	28	3	300	15	72.7	2.7285	0.5114	3.75	0.70
h7	28	3	1800	15.5	84.6	2.4848	1.8501	2.94	2.19
h8	28	3	2400	17.3	112.8	0.6043	1.7675	0.54	1.57
h9	28	3	1200	14	61.4	2.5772	0.7022	4.20	1.14
h10	28	3	1200	14.1	67.1	2.1011	0.5604	3.13	0.84
h11	29	3	1700	15.3	77.9	2.5545	1.0251	3.28	1.32
h12	29	3	1300	15.3	81.2	2.5702	1.0704	3.17	1.32
h13	29	3	2500	17.3	118.5	4.0724	1.4348	3.44	1.21
h14	29	3	2900	17.9	128.7	3.1004	1.3896	2.41	1.08
h15	29	3	1300	14.5	65.6	1.4886	0.9214	2.27	1.40
h16	30	3	1400	14.5	58.9	1.4653	0.4582	2.49	0.78
h17	30	3	800	13.2	56.4	1.54761	0.37485	2.74	0.66
h18	30	3	2000	14.6	75.5	3.43564	1.27311	4.55	1.69
h19	30	3	1200	13.9	55.8	1.82523	0.79142	3.27	1.42
h20	30	3	2500	17.5	120	4.66924	2.16846	3.89	1.81
Mean			1625	15.8	90.6	2.74587	1.29299	3.08	1.37
SD			766	1.69	28.1	1.31578	0.63812	1.02	0.42
CV			47.2	10.6	31.0	47.9	49.4	33.2	30.9

Table B.2. (continued)

I.D.	time (d)	NP (ug/L)	blood vol (ul)	length(cm)	mass(g)	gonad (g)	liver (g)	GSI (%)	HSI (%)
a1	15	10	500	15	76.7	4.5078	1.0109	5.88	1.32
a2	27	10	1800	16.2	86.5	1.53261	1.45122	1.77	1.68
a3	27	10	200	13.2	56.9	1.5434	0.9658	2.71	1.70
a4	27	10	2600	17	108.9	4.47443	1.70399	4.11	1.56
a5	27	10	1800	17.5	120.1	2.25501	3.03653	1.88	2.53
a6	27	10	1700	16	83.3	1.963	1.57929	2.36	1.90
a7	28	10	1600	17	110.6	5.8313	2.0188	5.27	1.83
a8	28	10	1100	16	93.3	5.035	1.9561	5.40	2.10
a9	28	10	2000	16.3	89.9	0.685	1.7822	0.76	1.98
a10	28	10	2100	16.4	93.4	2.3099	1.397	2.47	1.50
a11	28	10	3100	18.1	139.7	2.2068	2.5225	1.58	1.81
a12	29	10	1500	14.7	71.3	1.777	1.2115	2.49	1.70
a13	29	10	2000	15.5	75.1	3.143	0.836	4.19	1.11
a14	29	10	3100	18.5	143	4.4995	3.034	3.15	2.12
a15	29	10	2000	16.3	97.3	4.1581	1.413	4.27	1.45
a16	29	10	1800	14	59.8	1.0879	1.0664	1.82	1.78
a17	30	10	2100	15.2	74.5	3.46291	not weighed	4.65	NA
a18	30	10	1400	13.9	58	0.8713	0.91787	1.50	1.58
a19	30	10	1100	14.4	66.4	3.31897	1.2805	5.00	1.93
a20	30	10	700	14.9	74.6	3.227	1.488	4.33	1.99
Mean			1710	15.8	89.0	2.89450	1.61429	3.28	1.77
SD			757	1.42	25.0	1.49408	0.65662	1.54	0.32
CV			44.3	8.96	28.1	51.6	40.7	46.9	18.2

Table B.3. Concentrations of 17beta-estradiol (E2) in plasma from individual sexually mature male carp exposed to 4-nonylphenol (NP) Mean, standard deviation (SD), and coefficient of variation (CV) across three replicate determinations is presented. Method detection limit was 175 pg E2/ml. Values less than 175 pg E2/ml may not be accurate.

I.D.	E2 (pg/ml)	SD	CV	
A10	112	12.3	11.0	
A11	116	9.8	8.4	
A12	3374	144.4	4.3	Outlier
A13	191	26.0	13.6	
A14	217	41.9	19.3	
A15	142	2.3	1.6	Nominal Exposure Conc. 10 ug NP / L
A16	114	8.1	7.1	
A17	277	9.4	3.4	
A18	302	67.6	22.4	
A19	140	35.4	25.2	
A20	168	31.1	18.5	
A4	188	21.1	11.2	
A5	133	11.7	8.8	
A6	158	14.0	8.8	
A7	199	14.9	7.5	
A9	68	18.4	27.2	
Mean	168			
SD	63.2			
n	16			

I.D.	E2 (pg/ml)	SD	CV	
C1	88	76.7	87.5	
C10	272	25.8	9.5	
C11	179	27.3	15.3	
C12	128	29.2	22.7	
C13	185	39.8	21.5	
C14	420	19.4	4.6	
C15	181	12.9	7.1	
C16	196	21.7	11.1	
C19	301	20.5	6.8	
C2	142	100.5	71.0	
C20	119	11.0	9.2	
C21	177	18.6	10.5	
C3	221	103.9	47.1	
C4	687	47.4	6.9	
C5	136	11.9	8.7	
C6	249	35.4	14.2	
C7	107	5.6	5.2	
C8	184	22.0	12.0	
C9	174	23.9	13.7	
Mean	218			
SD	137.5			
n	19			

Nominal Exposure Conc.
Solvent Control

Table B.3. (continued)

I.D.	E2 (pg/ml)	SD	CV
D1	138	128.2	92.8
D10	145	29.4	20.3
D11	235	57.6	24.6
D12	133	30.8	23.1
D13	287	19.8	6.9
D14	209	29.2	14.0
D15	204	25.9	12.7
D17	156	23.3	14.9
D19	206	29.4	14.2
D2	91	45.1	49.3
D20	152	9.8	6.5
D3	188	86.8	46.2
D4	198	52.6	26.6
D5	102	22.6	22.2
D6	165	32.3	19.6
D8	158	27.4	17.3
D9	105	17.3	16.4
D9	238	42.5	17.8
Mean	173		
SD	52.1		
n	18		

Nominal Exposure Conc.
Control

I.D.	E2 (pg/ml)	SD	CV
E1	172	129.2	74.9
E10	110	9.3	8.5
E12	186	21.9	11.8
E13	228	45.2	19.8
E14	179	29.0	16.2
E15	314	17.7	5.6
E17	159	31.3	19.7
E18	269	20.4	7.6
E19	123	30.7	24.9
E20	160	8.4	5.2
E3	218	82.8	38.1
E4	161	22.3	13.9
E5	136	10.3	7.6
E6	298	5.7	1.9
E7	163	19.0	11.6
E8	217	17.9	8.2
E9	78	21.2	27.0
Mean	187		
SD	64.4		
n	17		

Nominal Exposure Conc.
1.0 ug NP / L

Table B.3. (continued)

I.D.	E2 (pg/ml)	SD	CV
F1	107	96.6	90.5
F10	208	16.4	7.9
F11	79	8.0	10.1
F12	2935	240.4	8.2
F13	255	36.9	14.5
F14	241	48.2	20.0
F15	163	12.4	7.6
F16	150	5.1	3.4
F17	327	21.5	6.6
F18	170	20.6	12.1
F2	113	95.5	84.7
F3	301	161.7	53.7
F4	136	8.3	6.1
F5	229	20.0	8.7
F6	232	26.8	11.6
F8	193	46.5	24.1
F9	174	41.3	23.7
F9	173	2.8	1.6
Mean	191		
SD	67.4		
n	17		

Female

Nominal Exposure Conc.
0.3 ug NP / L

I.D.	E2 (pg/ml)	SD	CV
G1	115	91.1	79.2
G10	166	10.1	6.1
G11	134	34.1	25.5
G12	261	30.4	11.7
G13	299	40.5	13.5
G14	180	25.6	14.2
G15	249	20.3	8.2
G16	364	10.6	2.9
G18	337	51.6	15.3
G19	171	28.1	16.5
G2	115	88.5	76.9
G3	147	12.1	8.2
G3	380	68.3	18.0
G4	331	15.3	4.6
G5	97	14.9	15.4
G6	155	51.2	33.0
G7	402	85.8	21.3
G8	122	24.5	20.0
G9	88	17.8	20.2
Mean	216		
SD	105.9		
n	19		

Nominal Exposure Conc.
0.1 ug NP / L

Table B.3. (continued)

I.D.	E2 (pg/ml)	SD	CV
H1	202	158.6	78.6
H10	100	3.3	3.3
H11	103	28.2	27.3
H12	276	19.3	7.0
H13	700	88.8	12.7
H14	159	23.0	14.5
H15	301	54.1	18.0
H16	262	10.1	3.9
H17	191	11.4	6.0
H18	298	60.1	20.2
H2	136	94.5	69.4
H20	357	43.4	12.1
H3	241	26.9	11.2
H4	169	13.1	7.8
H5	174	28.6	16.5
H7	306	21.9	7.2
H8	109	7.6	7.0
H9	108	10.1	9.4
Mean	233		
SD	142		
n	18		

Nominal Exposure Conc.
3.0 ug NP / L

Table B.4. Concentrations of testosterone (T) in plasma from individual sexually mature male carp exposed to 4-nonylphenol (NP) Mean, standard deviation (SD), and coefficient of variation (CV) across three replicate determinations is presented.
a = 1:30 dilution of plasma extract; b = 1:90 dilution of plasma extract

I.D.	T (pg/ml)	SD	CV	a,b mean
A10a	3613	245	6.8	3780
A10b	3948	399	10.1	
A11a	9194	715	7.8	9519
A11b	9844	977	9.9	
A12a	2222	329	14.8	2489
A12b	2756	339	12.3	
A14a	2616	272	10.4	2692
A14b	2768	72	2.6	
A15a	5711	258	4.5	5948
A15b	6184	924	14.9	
A17a	8673	933	10.8	9038
A17b	9404	290	3.1	
A18a	4120	276	6.7	4240
A18b	4361	305	7.0	
A5a	3372	113	3.4	4176
A5b	4981	560	11.2	
A6a	11610	4847	41.7	13624
A6b	15638	717	4.6	
Mean	6167			6167
SD	3745			3777
CV	61			61

Nominal Conc.
10 ug NP/L

I.D.	T (pg/ml)	SD	CV	a,b mean
C10a	11648	748	6.4	11246
C10b	10844	1576	14.5	
C12a	6742	222	3.3	6643
C12b	6544	186	2.8	
C13a	4983	224	4.5	6359
C13b	7736	930	12.0	
C14a	8384	40	0.5	8776
C14b	9168	150	1.6	
C15a	6187	394	6.4	6854
C15b	7521	399	5.3	
C17a	13555	890	6.6	13858
C17b	14161	1378	9.7	
C19a	8440	309	3.7	9001
C19b	9563	454	4.7	
C1a	10137	493	4.9	10492
C1b	10847	2968	27.4	
C4a	10342	1244	12.0	11010
C4b	11678	1292	11.1	
C9a	21227	341	1.6	24669
C9b	28110	2859	10.2	
Mean	10891			10891
SD	5403			5394
CV	50			50

Nominal Conc.
Solvent control

Table B.4. (continued)

I.D.	T (pg/ml)	SD	CV	a,b mean
D10a	9866	384	3.9	10990
D10b	12114	276	2.3	
D11a	3800	410	10.8	3709
D11b	3617	664	18.4	
D13a	4138	135	3.3	3897
D13b	3655	315	8.6	
D15a	3419	338	9.9	3199
D15b	2980	324	10.9	
D16a	2049	181	8.8	2302
D16b	2555	423	16.5	
D19a	7247	431	5.9	7496
D19b	7744	288	3.7	
D20a	8884	361	4.1	9807
D20b	10730	1036	9.7	
D2a	3229	638	19.8	3434
D2b	3640	520	14.3	
D3a	9122	166	1.8	8830
D3b	8538	885	10.4	
D9a	12651	303	2.4	12087
D9b	11523	419	3.6	
Mean	6575			6575
SD	3612			3669
CV	55			56

Nominal Conc.
Control

I.D.	T (pg/ml)	SD	CV	a,b mean
E12a	12705	676	5.3	14256
E12b	15807	324	2.0	
E14a	11698	343	2.9	12679
E14b	13659	533	3.9	
E15a	5700	320	5.6	5325
E15b	4950	963	19.4	
E16a	9865	882	8.9	8545
E16b	7225	201	2.8	
E18a	16274	992	6.1	18727
E18b	21180	1724	8.1	
E19a	11815	1038	8.8	13941
E19b	16067	664	4.1	
E2a	20741	608	2.9	24118
E2b	27495	3316	12.1	
E5a	10265	655	6.4	10311
E5b	10357	907	8.8	
E7a	3973	237	6.0	3961
E7b	3948	828	21.0	
E8a	1293	145	11.3	1767
E8b	2241	191	8.5	
Mean	11363			11363
SD	6909			6880
CV	61			61

Nominal Conc.
1.0 ug NP/L

Table B.4. (continued)

I.D.	T (pg/ml)	SD	CV	a,b mean
F10a	8329	428	5.1	8189
F10b	8049	339	4.2	
F11a	3639	335	9.2	3757
F11b	3874	392	10.1	
F12a	6060	466	7.7	5601
F12b	5142	445	8.7	
F13a	3435	110	3.2	3466
F13b	3497	398	11.4	
F15a	1558	359	23.1	1888
F15b	2219	203	9.2	
F16a	2443	208	8.5	2573
F16b	2704	163	6.0	
F17a	4758	89	1.9	4876
F17b	4993	666	13.3	
F3a	14078	1146	8.1	15991
F3b	17904	1541	8.6	
F6a	18393	583	3.2	20896
F6b	23398	682	2.9	
F7a	16777	846	5.0	18763
F7b	20748	1633	7.9	
Mean	8600			8600
SD	7090			7175
CV	82			83

Nominal Conc.
0.3 ug NP/L

I.D.	T (pg/ml)	SD	CV	a,b mean
G12a	1544	98	6.3	1775
G12b	2007	351	17.5	
G13a	5172	400	7.7	4802
G13b	4433	494	11.1	
G15a	7465	435	5.8	7857
G15b	8250	426	5.2	
G18a	6000	148	2.5	6084
G18b	6168	259	4.2	
G19a	6875	380	5.5	6713
G19a	6551	923	14.1	
G1a	14060	988	7.0	13986
G1b	13912	1767	12.7	
G5a	6290	945	15.0	5650
G5a	5010	477	9.5	
G5b	7229	377	5.2	5955
G5b	4681	224	4.8	
G7a	13592	418	3.1	17478
G7b	21364	733	3.4	
G9a	5260	528	10.0	4866
G9b	4472	404	9.0	
Mean	7517			7517
SD	4755			4681
CV	63			62

Nominal Conc.
0.10 ug NP/L

Table B.4. (continued)

I.D.	T (pg/ml)	SD	CV	a,b mean
H11a	2642	312	11.8	2802
H11b	2961	230	7.8	
H12a	7224	116	1.6	6498
H12b	5772	853	14.8	
H13a	2196	142	6.5	2387
H13b	2578	398	15.4	
H16a	943	250	26.5	1711
H16b	2479	185	7.5	
H18a	12860	167	1.3	14760
H18b	16661	1568	9.4	
H1a	3281	280	8.5	3223
H1b	3166	133	4.2	
H2a	10999	212	1.9	11318
H2b	11638	555	4.8	
H3a	5303	379	7.1	5672
H3b	6041	146	2.4	
H8a	4727	388	8.2	4767
H8b	4807	162	3.4	
Mean	5904			5904
SD	4355			4419
CV	74			75

Nominal Conc.
3.0 ug NP/L

Table B.5. Concentrations of vitellogenin (VTG) in plasma from individual sexually mature male carp exposed to 4-nonylphenol (NP). Mean across three replicate determinations is presented. Value reported is for the dilution which yielded a %-bound closest to 50%.

The method detection limit (MDL) was 1 ug VTG/ml.

Values less than 1.0 ug VTG/ml may not be accurate.

I.D.	VTG (ug/ml)	I.D.	VTG (ug/ml)	I.D.	VTG (ug/ml)
A10	0.2	E10	0.7	H10	0.2
A11	0.7	E12	0.1	H12	0.8
A12	3.7	E13	0.7	H13	0.5
A13	1.6	E15	0.2	H14	0.4
A14	0.5	E17	0.9	H15	52
A15	0.5	E18	5.8	H16	0.2
A16	2.7	E19	1	H18	12.3
A18	0.9	E2	0.2	H2	0.4
A2	0.1	E20	10.8	H20	0.1
A4	0.5	E3	0.2	H3	0.3
A6	0.8	E5	0.1	H7	3.2
A9	0.7	E8	1.1	H8	0.3
C10	1.4	F11	1.4		
C13	0.3	F12	>> 50		
C14	2.2	F13	3.6		
C16	0.3	F15	1.8		
C17	0.6	F16	1.9		
C19	1.6	F18	0.2		
C2	0.1	F2	0.4		
C20	0.7	F3	7.3		
C3	0.5	F4	3.6		
C8	0.3	F5	0.9		
C9	0.3	F6	0.5		
D10	0.5	F8	0.3		
D12	8.9	G1	0.5		
D13	0.7	G11	2.2		
D15	0.5	G12	1.7		
D17	50	G13	4.5		
D19	14.2	G15	50		
D2	1.6	G17	3.2		
D20	1.3	G18	0.1		
D3	0.3	G2	1		
D4	1.1	G4	0.1		
D5	0.2	G6	3.8		
D8	0.6	G8	0.3		
D9	0.8	G9	0.6		

Nominal Conc.
A = 10 ug NP/L
C = Solvent control
D = Control
E = 1.0 ug NP/L
F = 0.3 ug NP/L
G = 0.1 ug NP/L
H = 3.0 ug NP/L

APPENDIX C

VITELLOGENIN ELISA PLASMA INTERFERENCES EXPERIMENT

Background

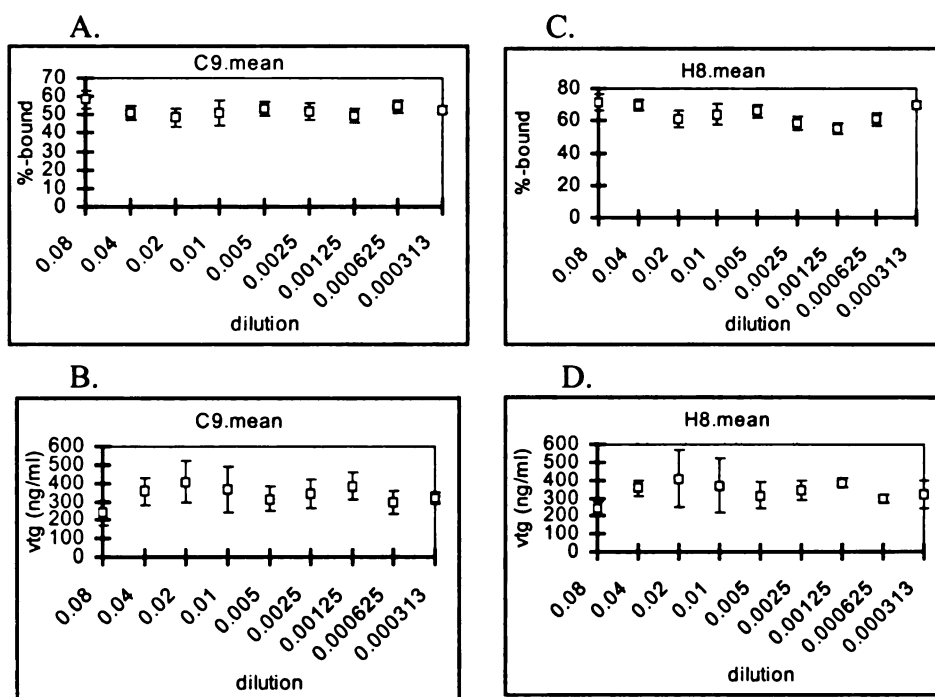
At high concentrations, plasma proteins have the potential to interfere with the vitellogenin (VTG) ELISA. Plasma samples are routinely diluted to avoid such interferences. When VTG concentrations are relatively high, such dilution does not restrict accurate quantitation. When trying to detect relatively low concentrations of VTG, however, it is desirable to dilute plasma samples as little as possible in order to lower the method detection limit (MDL). The objective of this experiment was to determine the minimum amount of plasma dilution which could be used while still avoiding interference from other plasma constituents.

Experimental Design

Plasma samples (C9 and H8), which had been previously shown to have non-detectable concentrations of VTG, were diluted in buffer containing a constant concentration of VTG standard (268 ng VTG/ml). This was selected as a concentration expected to yield approximately 50% binding in the VTG ELISA. Nine dilutions of each sample were prepared using 2-fold serial dilution. This yielded final plasma dilutions of 1:12.5 (0.08), 1:25 (0.04), 1:50 (0.02), 1:100 (0.01), 1:200 (0.005), 1:400 (0.0025), 1:800 (0.00125), 1:1600 (0.000625), and 1:3200 (0.000313). Each dilution was analyzed in triplicate using the VTG ELISA procedure described by Snyder (2000). Results expressed in both %-bound and final calculated concentration of VTG were compared to determine at which dilutions plasma interferences may occur.

Results

Fig. C.1. VTG ELISA results for plasma samples (C9 and H8) diluted in buffer containing 268 ng VTG/ml. Mean of three replicate determinations $\pm$ one standard deviation is presented. Plots A and C depict responses expressed as %-bound for dilutions of samples C9 and H8, respectively. Plots B and D depict responses expressed as final calculated concentration of VTG for dilutions of samples C9 and H8, respectively. Dilution refers to the ratio of whole plasma to the total volume of dilute plasma sample (ex. 0.08 = 1 μ l plasma / 12.5 μ l dilute sample).



Among the dilutions tested, only the 0.08 dilution (1:12.5) appeared to give an ELISA response which was markedly different from that of the other dilutions tested. Dilutions greater than 1:20 appeared to be sufficient to prevent marked plasma interferences.

Reference:

Snyder, E.M., 2000. Use of fish as bioassay organisms to assess wastewater effluents for reproductive endocrine modulating chemicals. Ph.D. Dissertation. Michigan State University, East Lansing, MI, USA (in preparation).

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