An Effects-Based Expert System to Predict Estrogen Receptor Binding Affinity for Food Use Inert Ingredients & Antimicrobial Pesticides: Application in a Prioritization Scheme for Endocrine Disruptor Screening

EPA, ORD, NHEERL, Mid-Continent Ecology Division

&

EPA, Office of Pesticide Programs

OECD Endocrine Testing & Assessment Conceptual Framework

- Level 1 - Sorting and prioritizing with existing data and/or (Q)SARs
- Level 2 - In vitro assays to provide mechanistic data
- Level 3 - In vivo assays providing data about single endocrine mechanisms and effects
- Level 4 - In vivo assays providing data about multiple endocrine mechanisms and effects
- Level 5 - In vivo assays providing data about endocrine and other effects

(OECD, 2004b)

Perspectives to (Q)SAR Development & Application

- Development and use of (Q)SARs in a regulatory context requires clear problem definition
  - The purpose of the (Q)SAR application must be well-defined
    - priority setting to support screening decisions is a very different application from chemical-specific risk assessments; each use will have different criteria for regulatory acceptance of the estimated data
- The interpretation of a (Q)SAR output for a specified endpoint is based on underlying toxicological knowledge and data upon which the model is derived
  - Optimal state: using toxicity data (as training set for developing a QSAR model) based on a well-defined endpoint in a well-defined assay, i.e., a model is only as good as the data used to build it

OECD Principles for QSAR Validation:

The importance of Transparency of the Modeling Approach and the data it is based upon, as well as Utility of the model for a Specified Application

- Well-defined biological endpoint that is basis of building the predictive model
- A mechanistic interpretation linking chemical structure to activity (endpoint) being predicted
- Define the chemical domain that the model covers
- Appropriate measures of goodness of fit, robustness, ability to predict
- An unambiguous algorithm

(OECD, 2004a)

A (Q)SAR-Based Expert System to Predict Estrogen Binding Affinity

- Application for use in a prioritization scheme in the context of EDSTAC (USEPA, 1998a) and SAB/SAP (USEPA, 1999) recommendations
- The application is focused on data poor chemicals, i.e., those without enough information to determine if Tier 2 testing required; the goal is to prioritize which chemicals should go first into Tier 1
- System developed using OECD QSAR Validation principles (transparent; mechanistic)
- Model applicability domain –
  - food use pesticide inert ingredients
  - antimicrobial pesticides
- Development benefitted from two OECD peer consultations
  - May, 2008 Structural Alert Workshop (OECD, 2009b)
  - February, 2009 Expert Consultation to Evaluate an Estrogen Receptor Binding Affinity Model for Hazard Identification (OECD, 2009a)

OECD Expert Consultation Findings

- Generally supportive & recommended:
  - Expanded description of the mechanistic background and interpretation of the model, including release of additional training set data
  - Enhanced clarification of the model's domain boundaries to facilitate determining the degree to which other regulatory inventories overlap with the model's applicability domain
  - Automation of the expert system rules and implementation of the system in the OECD QSAR Application Toolbox
  - Future efforts to expand the model's domain to additional chemical inventories ; and
  - Noted that increased understanding of the relationship between ER binding affinity and in vivo effects could enhance predictions at higher levels of biological organization

(OECD Draft Report, 2009a –released recently by OECD with no modifications http://appli1.oecd.org/olis/2009doc.nsf/linkto/env-jm-mono(2009)33)

White Paper submitted to EPA Science Advisory Panel (SAP)

Incorporated the OECD Expert Consultations Recommendations

- Expanded description of the chemical structure space associated with the two chemical inventories of interest
- Expanded discussion of the mechanistic basis for interpreting and measuring low affinity binding to the ER and the approach for establishing a training set reflective of the inventories of interest
- A broader discussion of the strategy for developing the training sets for the two inventories and summary of findings
- A more detailed description of the expert system's rules hierarchy by using a system of decision trees

White Paper for EPA SAP also summarized on-going efforts & next steps

- Automating the expert system's rules
  - OECD QSAR Application Toolbox
- Evaluating the extent of interspecies differences in ER binding affinity for chemicals in the two inventories
  - Trout ER vs Human ER
- Expand Expert System to additional inventories; expand to additional endpoints, e.g., using HTPS data

Mechanistic Basis of the ER binding Expert System

Application of OECD Principles

Desired Outcomes:

- Transparency
  - Can the QSAR estimate be explained mechanistically?
  - How reasonable is the estimate compared with data for similar chemicals ?

- Usefulness
  - Are the predictions applicable to all the chemicals of concern?
  - Does the model/expert system answer the regulatory question?

Mechanistic Basis

(4 aspects were discussed in SAP review)

1)ER Binding Affinity: An Indicator of Potential Reproductive Effects

- ER-mediated reproductive impairment Adverse Outcome Pathway

2)ER Binding Domain

- Knowledge/theories of chemical-receptor interactions
  - ER sub-pockets

3)The Regulatory Chemical Domain

- Characterizing the food use inerts (FI) and antimicrobial pesticides (AM) inventory chemicals
- Building from existing information to strategically pick chemicals to expand the knowledge-base in an efficient and targeted manner

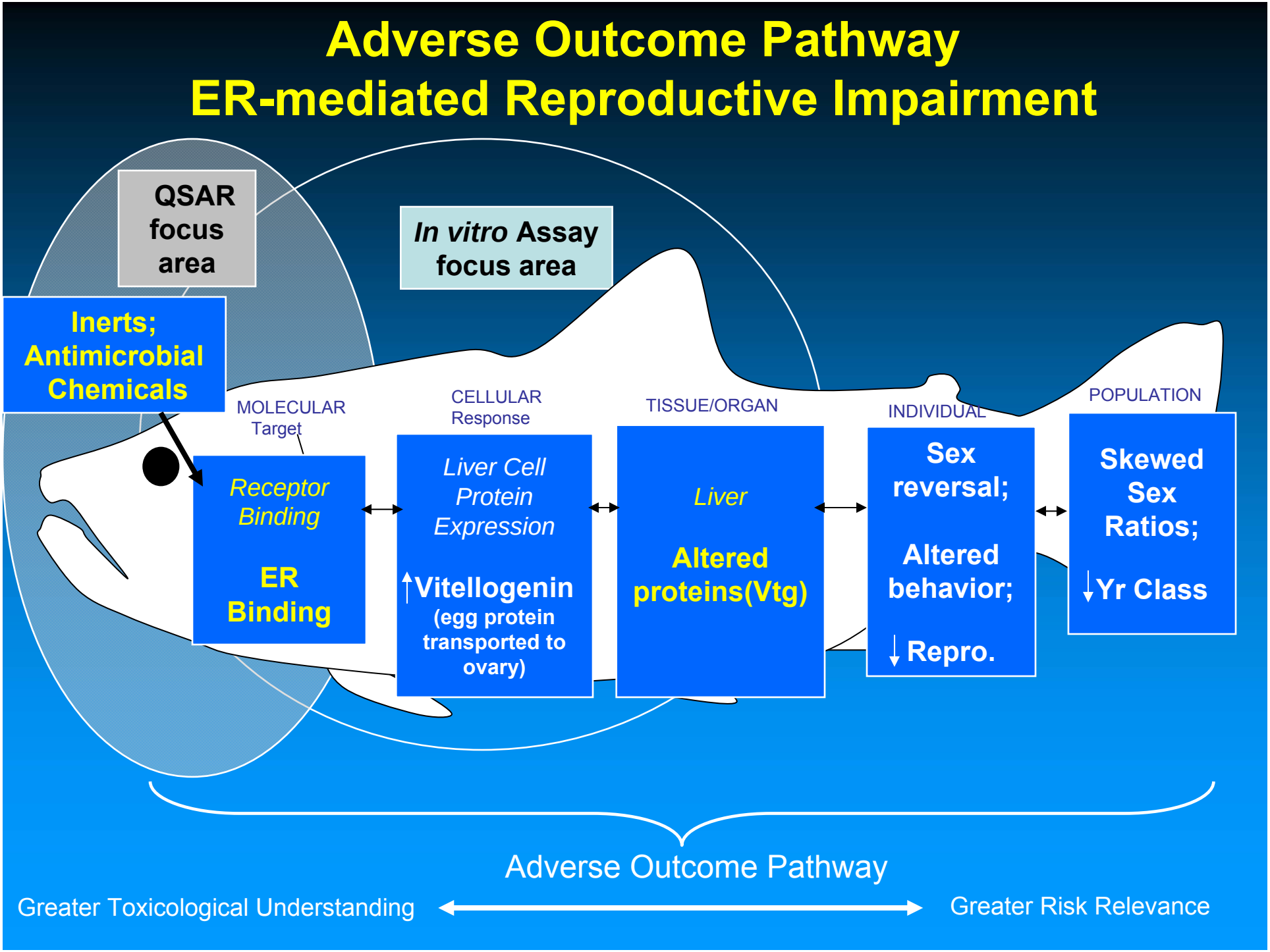
4)The Receptor Binding Assay Domain

- Optimizing in vitro assays considering physical-chemical properties of inventory chemicals

1) ER Binding Affinity: An Indicator of Potential Reproductive Effects

Mechanistic linkage exists between the risk assessment endpoint (ER-mediated reproductive impairment) and the hazard identification endpoint (ER binding)

Adverse Outcome Pathway
ER-mediated Reproductive Impairment



ER Binding Affinity: An Indicator of Potential Reproductive Effects

Mechanistic linkage exists between the risk assessment endpoint (ER-mediated reproductive impairment) and the hazard identification endpoint (ER binding)

Foundation of the assays used to build the Expert System is an adverse outcome pathway ending in reproductive impairment

- The molecular initiating event of the pathway is identified (ER binding)
- The expert system identifies which chemical structures can initiate the pathway
    - Pathway context provides conceptual model useful for generating testable hypotheses
    - Pathway context provides decision-making rationale for the regulatory community

ER-mediated Adverse Outcome Pathway:

- Area of focus consistent with legislative directive
- Chemical binding to the ER is known to have potential to cause adverse effects
- Evidence existed that diverse chemical structures bind ER

Mechanistic Basis of the Expert System to Predict Relative Estrogen Receptor Binding Affinity

(4 aspects were discussed in SAP review)

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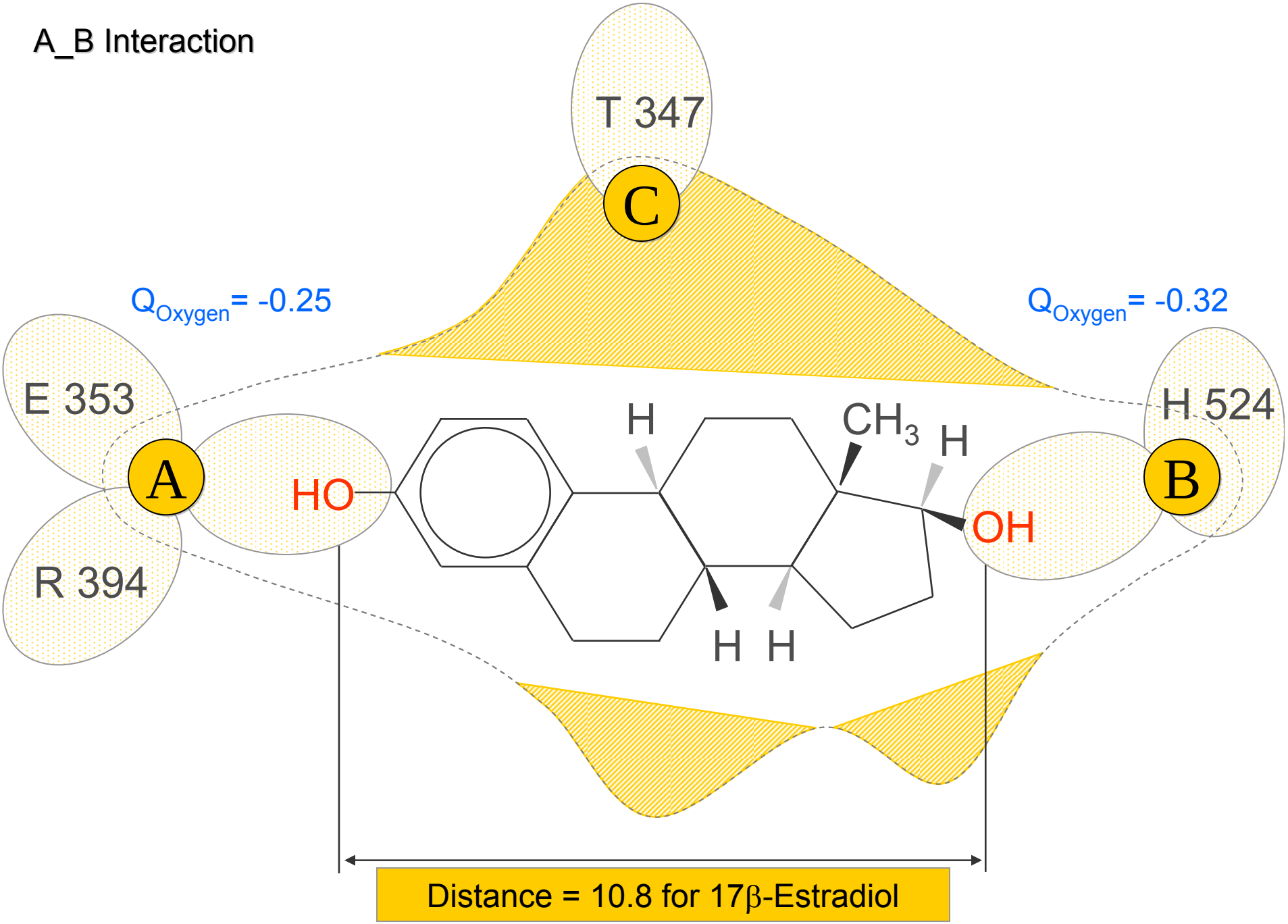
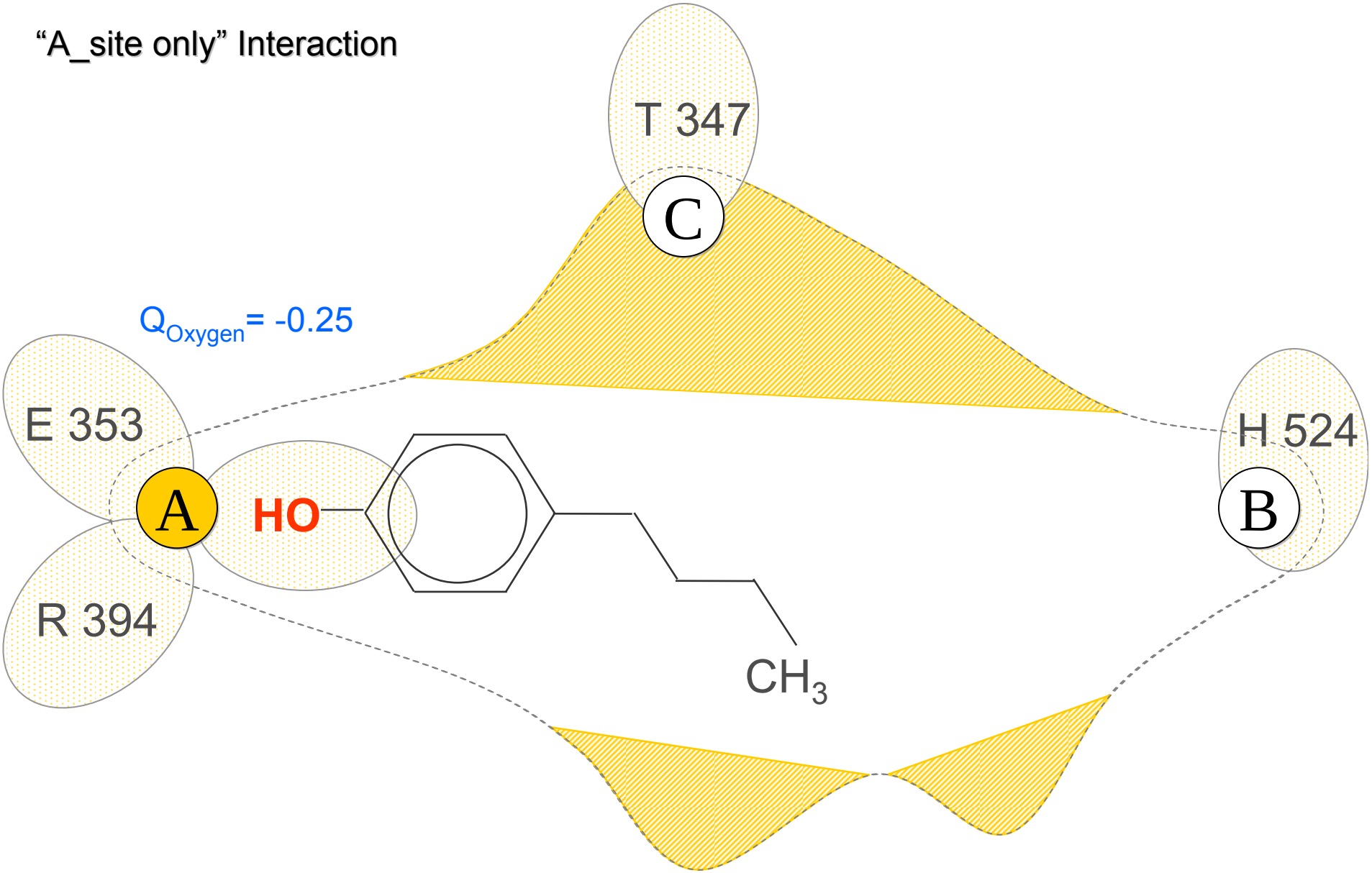
A_B Interaction



J. Katzenellenbogen

“A_site only” Interaction



J. Katzenellenbogen

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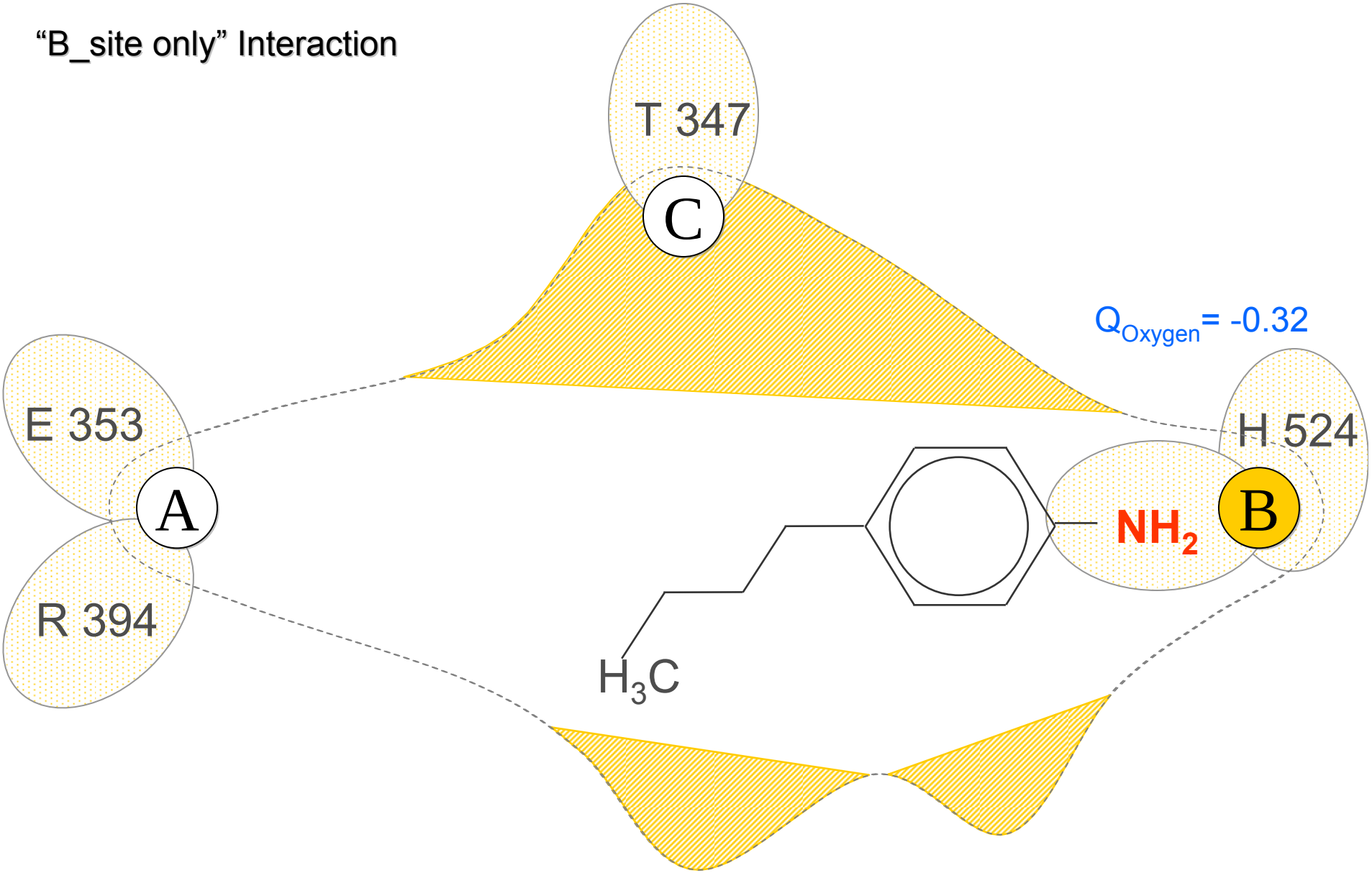

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Apply knowledge/theory of
2) ER Binding Domain
to
3) The Regulatory Chemical Domain

(continuing to seek **MECHANISTIC** *understanding)*

Hypothesize ER interactions of Inventory Chemicals

Inert ingredients and antimicrobial pesticides are non-steroidal and do not contain multiple H-bonding groups at distance needed for steroid-like interactions

Hypotheses:

- Any pesticide inert or antimicrobial that does bind ER will do so through an interaction mechanism that results in low affinity binding
- Only a small % of these chemicals are likely to bind ER
- A chemical group approach will facilitate regulatory application
- Chemicals can be grouped based on how they interact with the ER (within specific ER sub-pockets)

3) The Regulatory Chemical Domain

(Q)SAR Principles call for defining the **model domain** in terms of the chemical structures used to create the model

- Usefulness of a (Q)SAR model (expert system) is evaluated by comparing domain (chemical coverage) of the expert system to the regulatory chemical domain.

Most (Q)SAR models do not use a specific regulatory inventory to develop the model domain

This ER expert system provides estimates of ER binding derived from a knowledge-base specifically developed to cover the inventories of regulatory interest:

- inert ingredients in pesticides used on crops (FI)
- antimicrobial pesticides (AM)

Mechanistic Basis of the Expert System to Predict Relative Estrogen Receptor Binding Affinity

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The Receptor Binding Assay Domain

Well-Defined Endpoint: I. Biology

Focus on molecular initiating event (ER binding) within context of ER-mediated adverse outcome pathway

Optimize bioassay methods for FI and AM chemicals (chemical properties) to detect low affinity ER interactions:

1) rtER binding is assessed using a standard competitive binding assay optimized for FI and AM chemicals;
   - chemicals are tested until binding displacement is observed or solubility in assay media, whichever comes first, to determine any potential to bind ER

2) ER binding curves are interpreted by assaying for gene expression in a higher-order assay

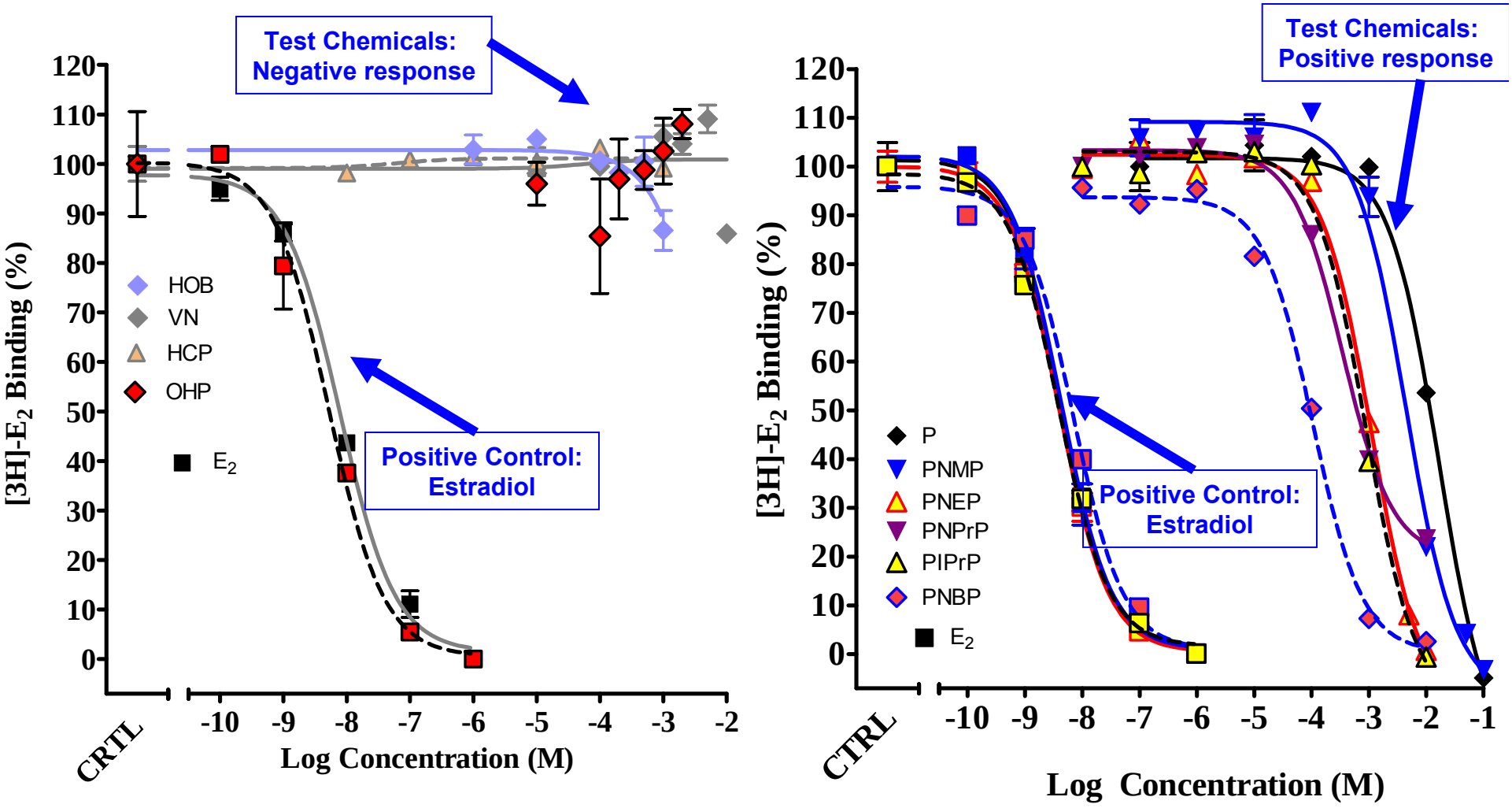
   ER-mediated vitellogenin mRNA production in metabolically competent trout liver slices;

3) additional experiments to verify competitive binding (e.g., Ki, dosimetry) are done as needed (e.g., charged alkylaromatic sulfonic acids)

Data Example - primary *In vitro* assay used :

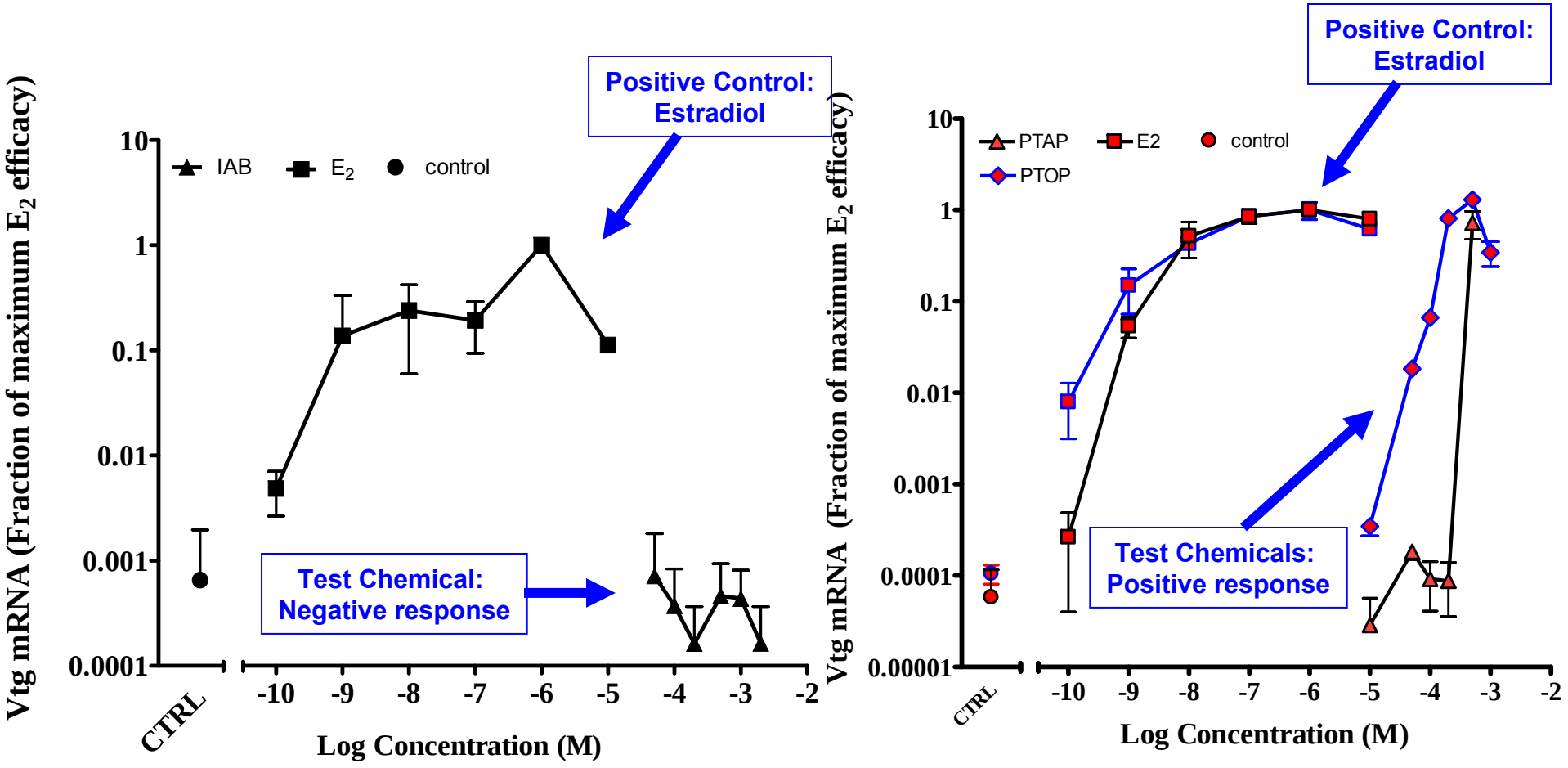
Estrogen Receptor Binding Displacement Assay

Cyto rtER



RBA = relative binding affinity; (a ratio of measured chemical affinity for the ER relative to 17-beta-Estradiol = 100%)
Log Kow = Log of octanol/water partition coefficient); indicator of lipophilicity

Data example – Confirmatory *in vitro* Assay:
Gene Activation



The ER Binding Assay Domain
Well-Defined Endpoint: II. Chemical Dosimetry

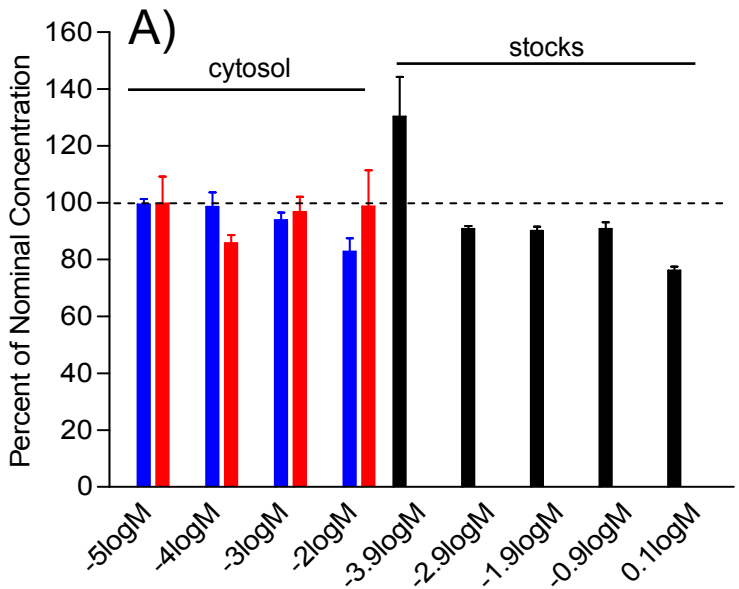
Additional information is gathered to better understand chemical behavior in the assays.

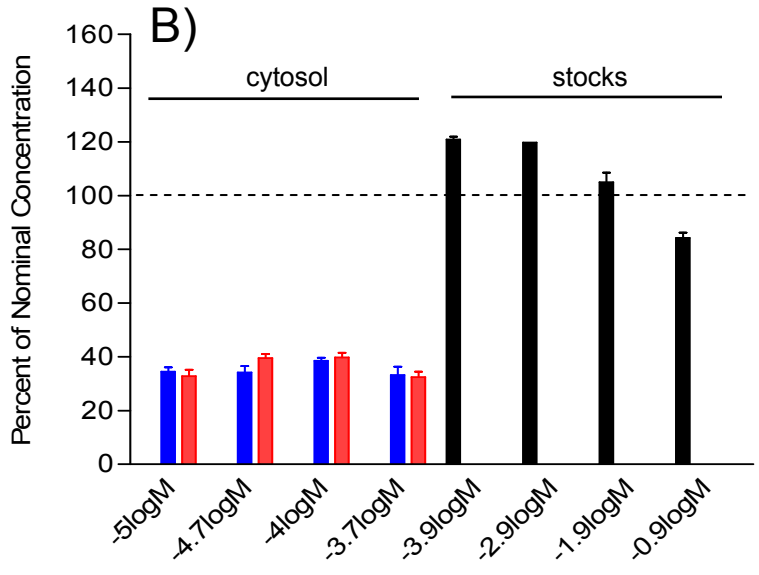
1) Chemical purity

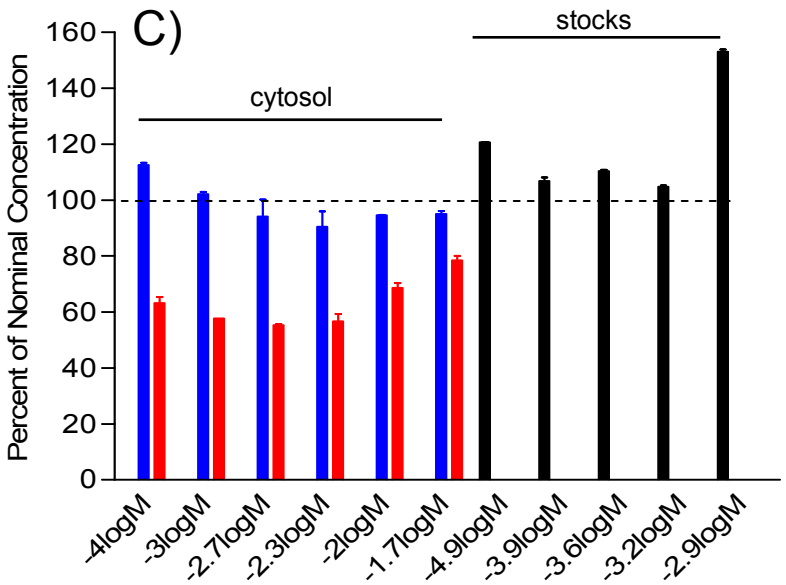
2) Metabolism
- Is the test system used for collection of empirical data capable of xenobiotic metabolism?
- If so, is activity (or lack of activity) due to parent chemical or a metabolite?

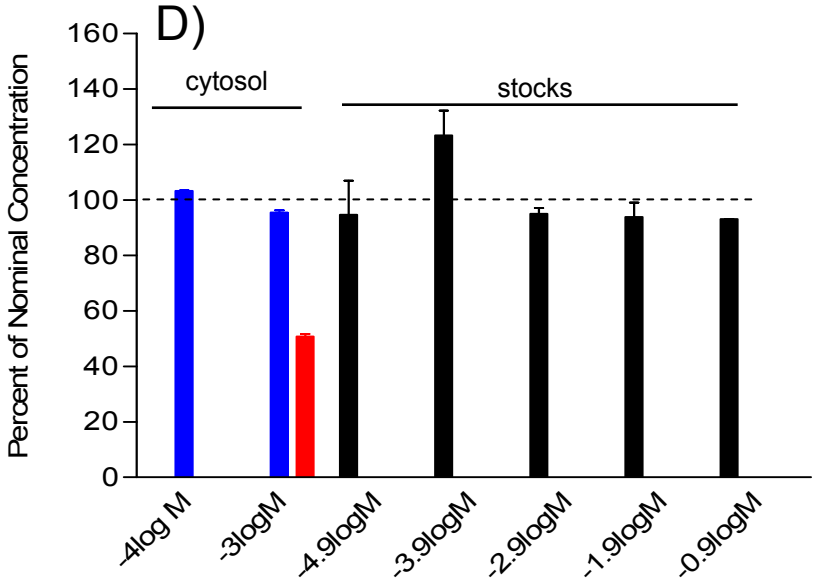
3) Bioavailability of the test chemical in the assay
- Rate of chemical 'disappearance' within the system (e.g. hydrolysis; partitioning to surfaces in assay system)
- Chemical solubility
  - Freely dissolved vs. bound and unavailable

Chemical Identity and Concentration Measured in ER Binding Assays

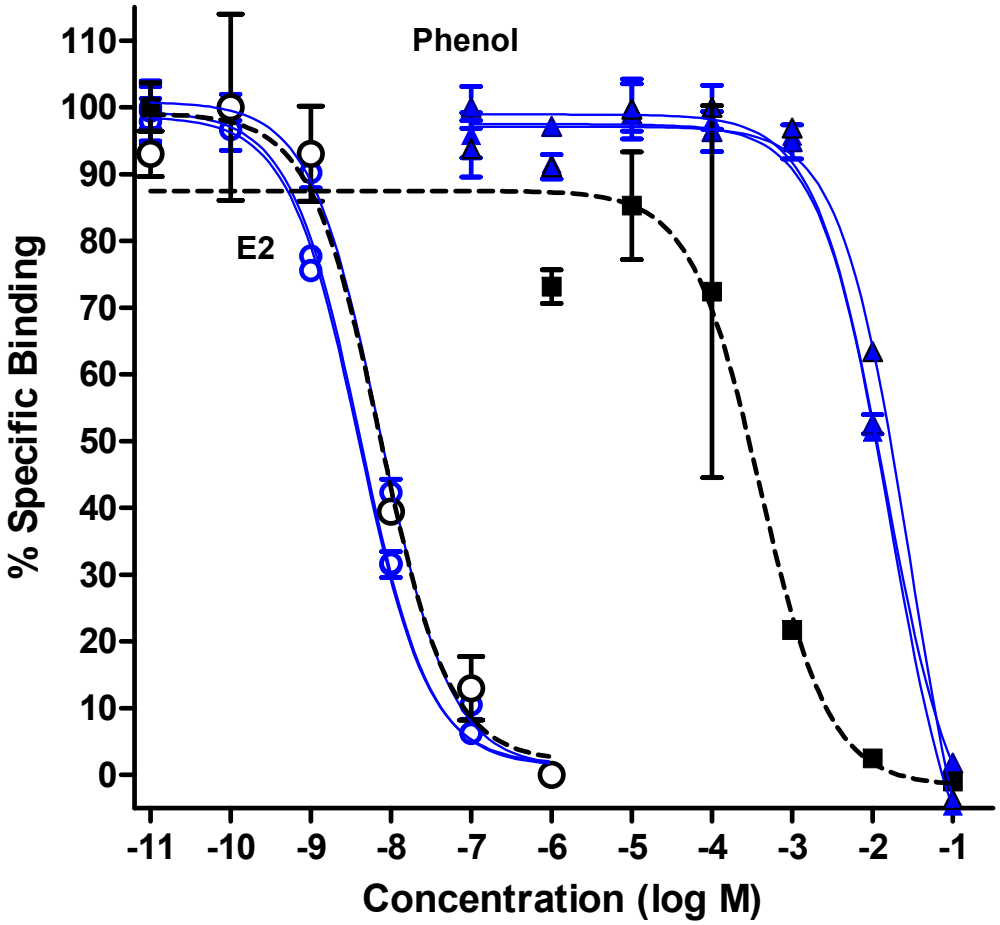








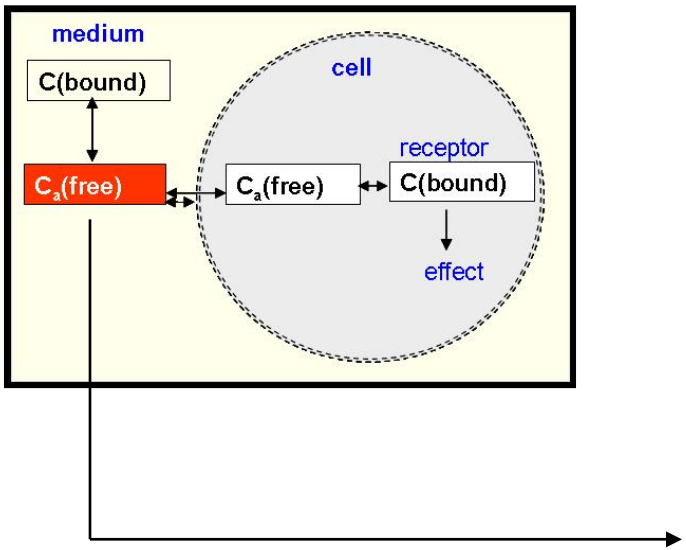
Assay total protein affects chemical bioavailability; increased assay total protein requires greater applied chemical to achieve the chemical free fraction needed to displace the endogenous ligand from the ER.

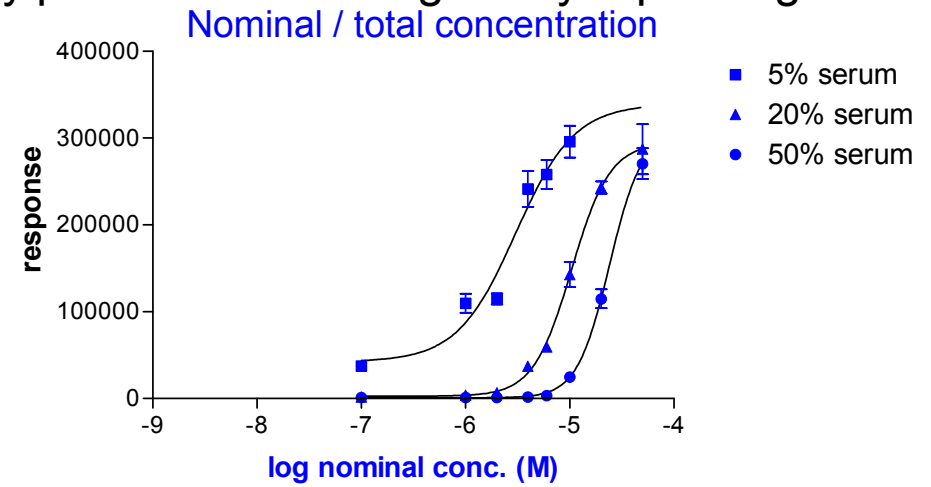


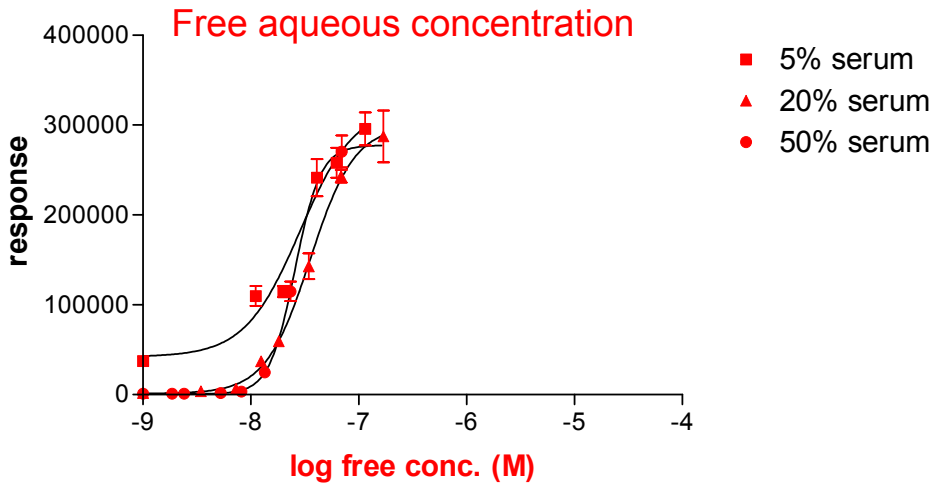
	rec rtERα	cyto rtER
E2	○	○
Phenol	■	▲
Total Protein (mg/mL)	0.01	4
RBA (%)	0.0025	0.000026

Assay total protein affects chemical bioavailability; Total chemical concentration must be increased as assay total protein increases to achieve a constant free fraction of chemical needed to achieve response (ER-mediated gene activation)

Total vs. free concentration of *p*-octylphenol in an estrogenicity reporter gene assay







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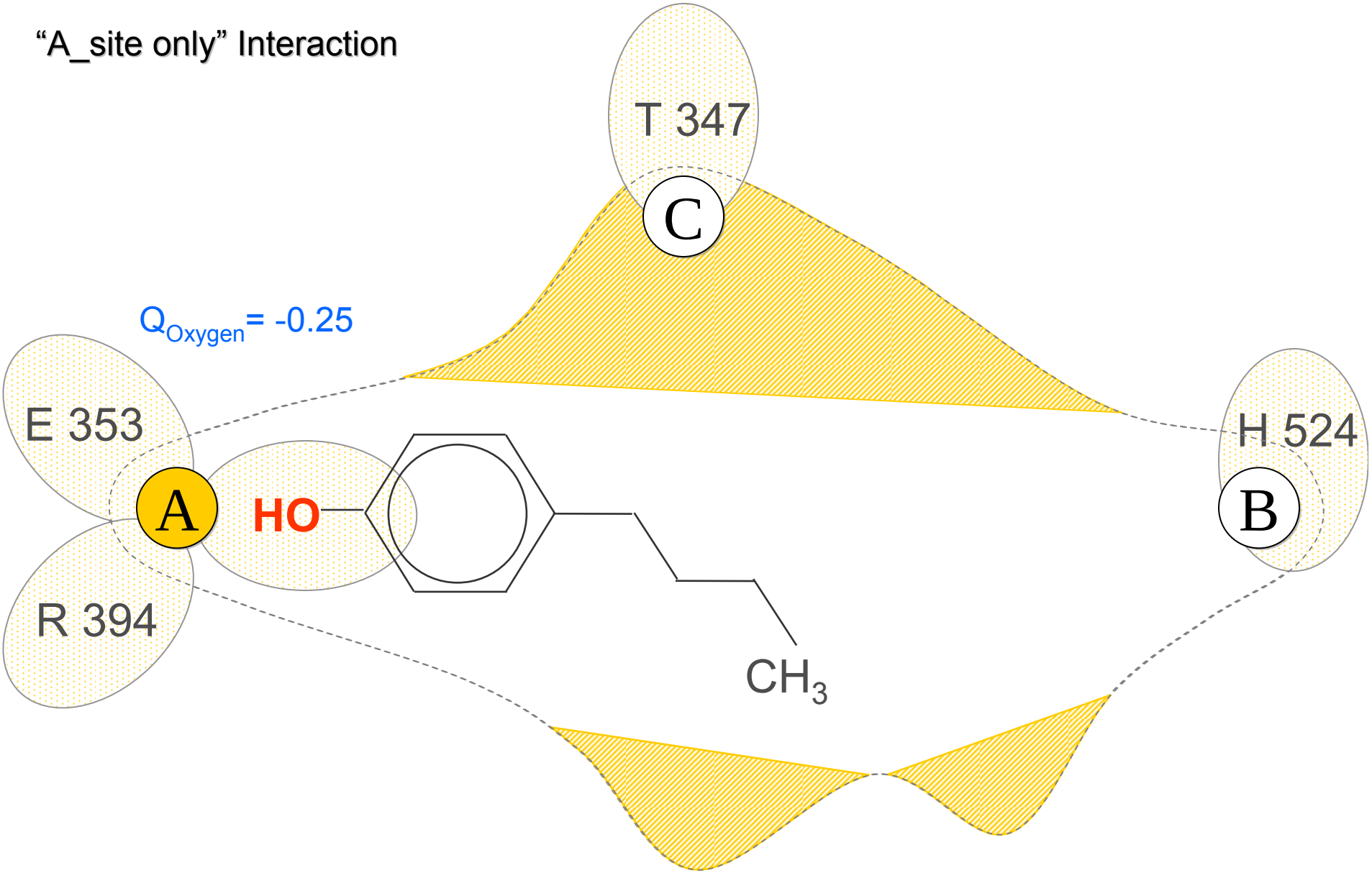
Regulatory Inventories of Interest and Expert System Modeling Domain

- Using mechanistic understanding of ER Binding Domain, Regulatory Chemical Domain, and ER Binding Assay Domain, to expand the expert system knowledge base (**model applicability domain**) to specifically cover:
  - Inert ingredients in pesticides used on food crops; FI ~400 chemicals
  - Antimicrobial pesticides; AM ~200 chemicals

- Coding knowledge gained into systematic logic rules

“A_site only” Interaction



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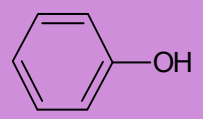
ER Binding **Site A** Homologous Series

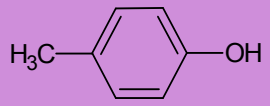
4-n-Alkylphenols

C0



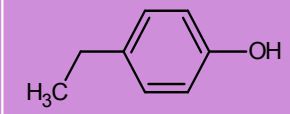
Log Kow = 1.50

C1



Log Kow = 1.97

C2



Log Kow = 2.47

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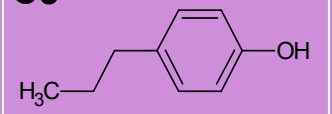
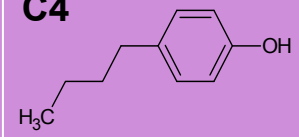


Log Kow = 3.20

C4



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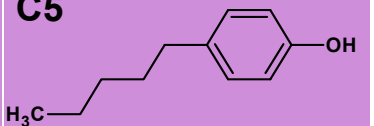
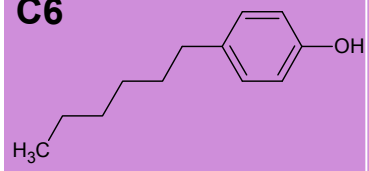
C5



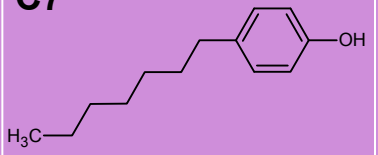
Log Kow = 4.06

C6



Log Kow = 4.62

C7



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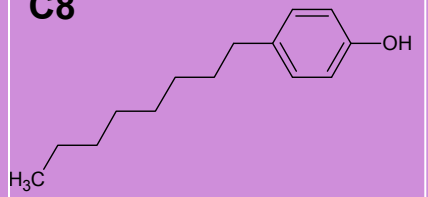
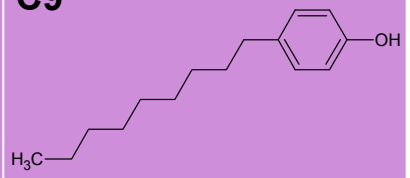


Log Kow = 5.68

C9

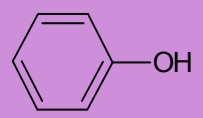


Log Kow = 5.76

Note: pink shading indicates RBA > 0.00001%; grey shading indicates RBA < 0.00001%

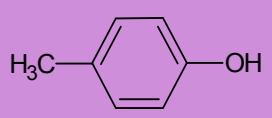
4-t-Alkylphenols were also assayed

C0



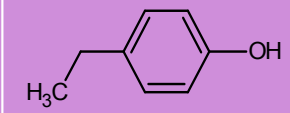
Log Kow = 1.50

C1



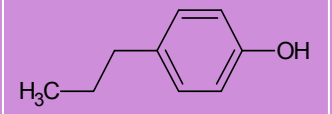
Log Kow = 1.97

C2



Log Kow = 2.47

C3



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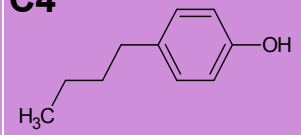

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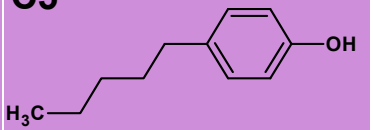
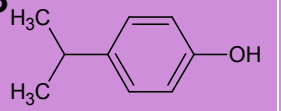


Log Kow = 4.06

C3



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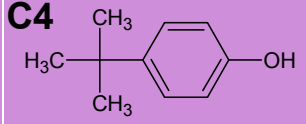

Log Kow = 3.31

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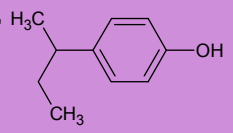

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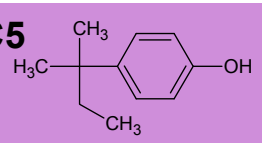

Log Kow = 3.83

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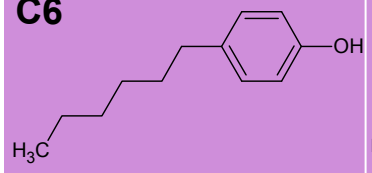
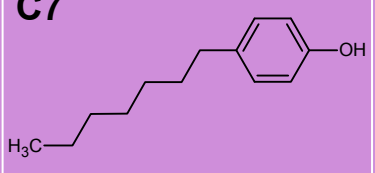


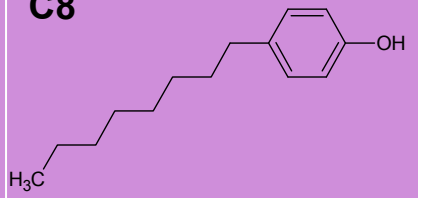
Log Kow = 4.62

C7



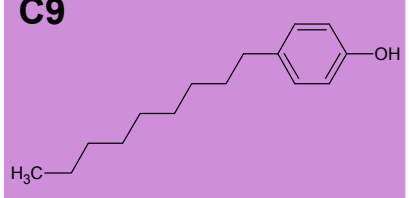
Log Kow = 4.15

C8



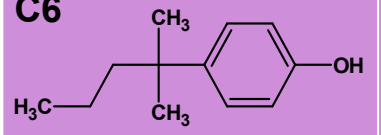
Log Kow = 5.68

C9



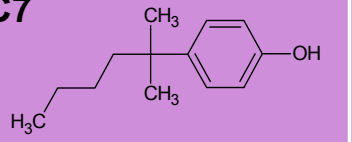
Log Kow = 5.76

C6



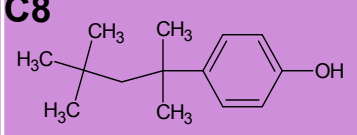
Log Kow = 4.36

C7



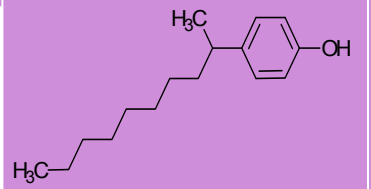
Log Kow = 4.89

C8



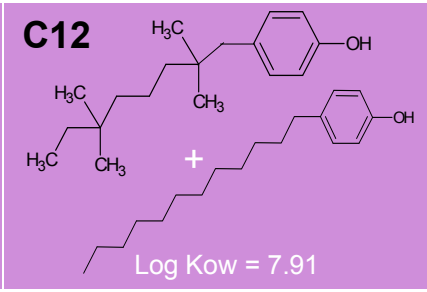
Log Kow = 5.16

C10



Log Kow = 6.61

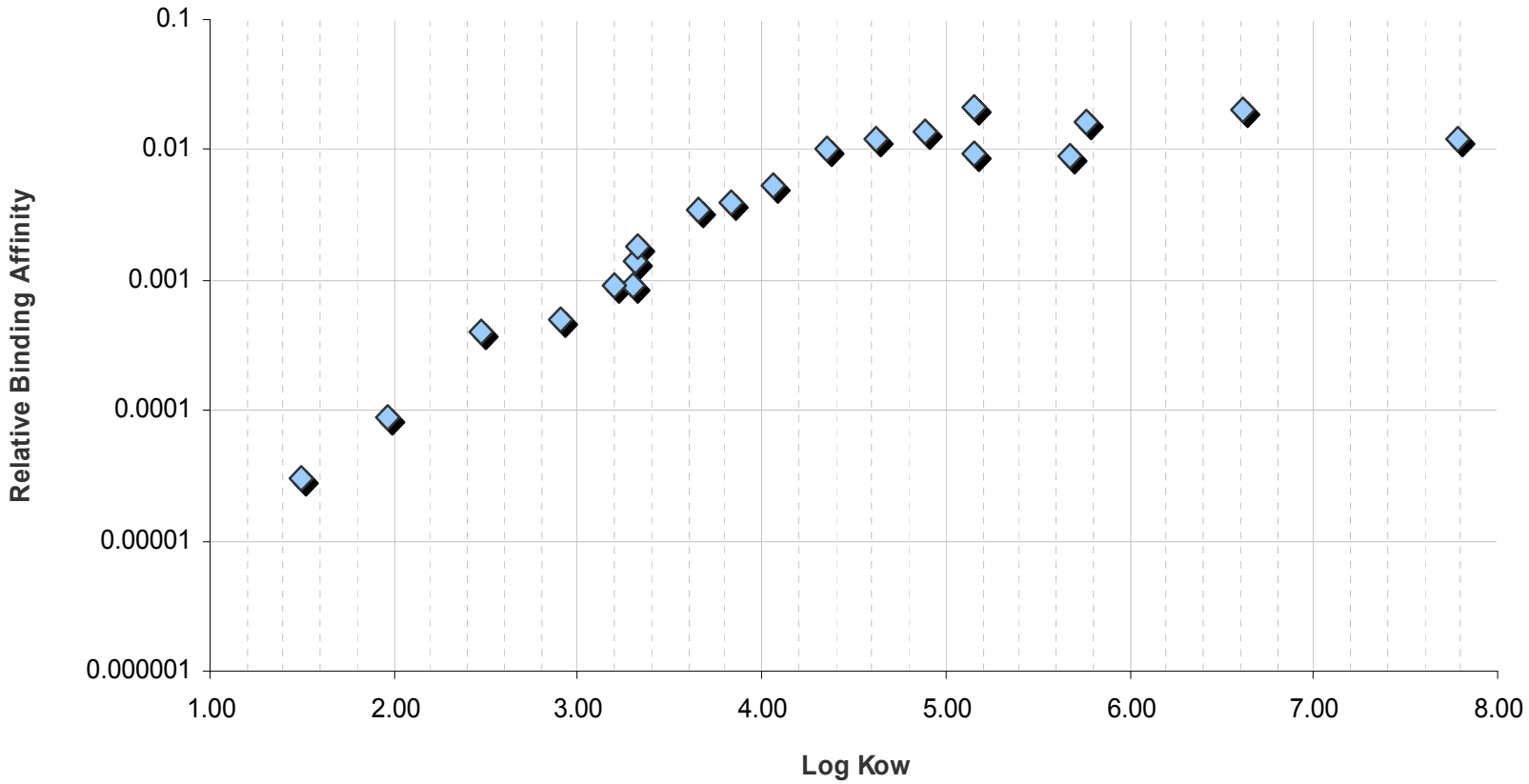
C12



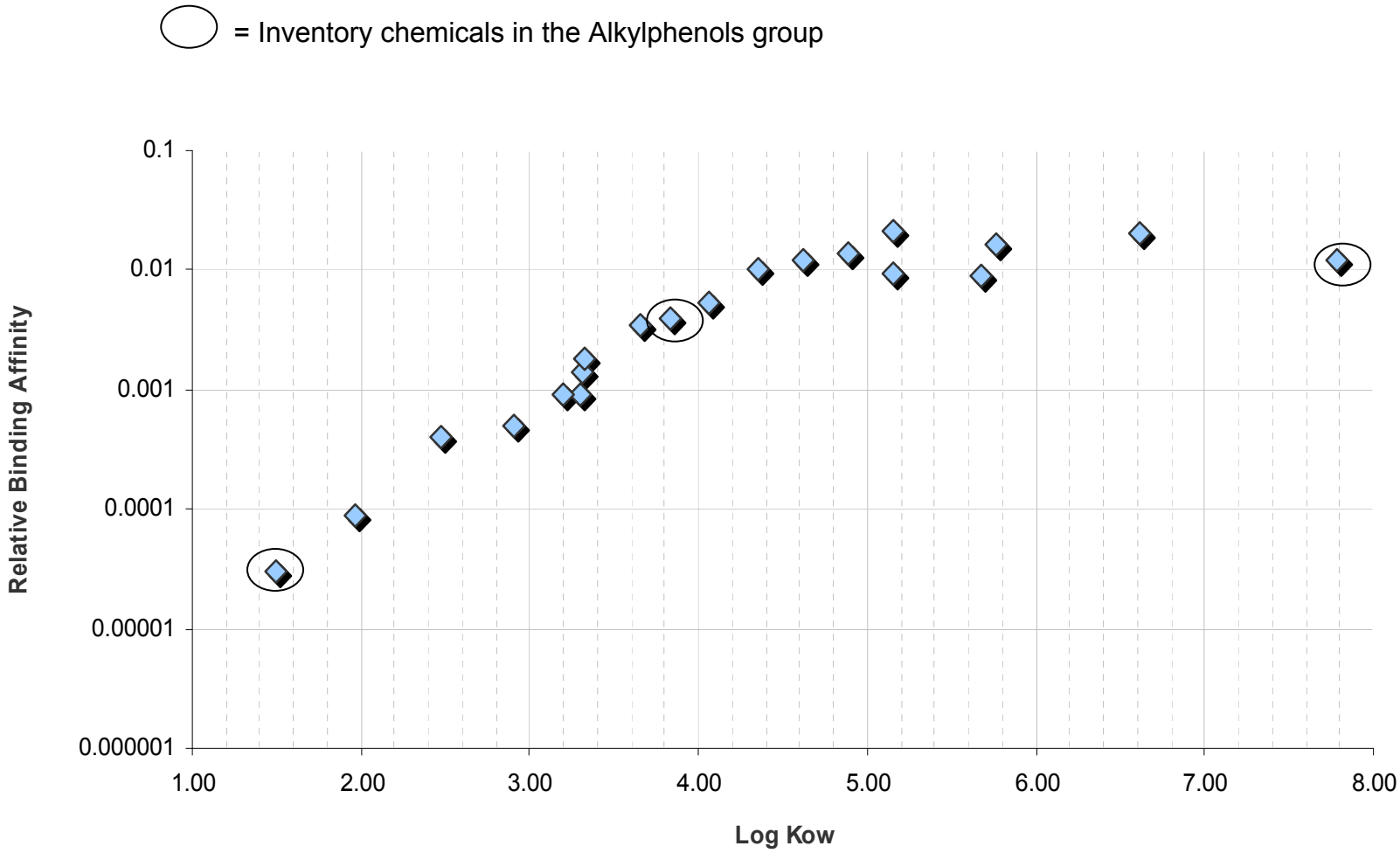
Log Kow = 7.91

Site A

Relationship between Log Kow and RBA for 4-alkylphenols

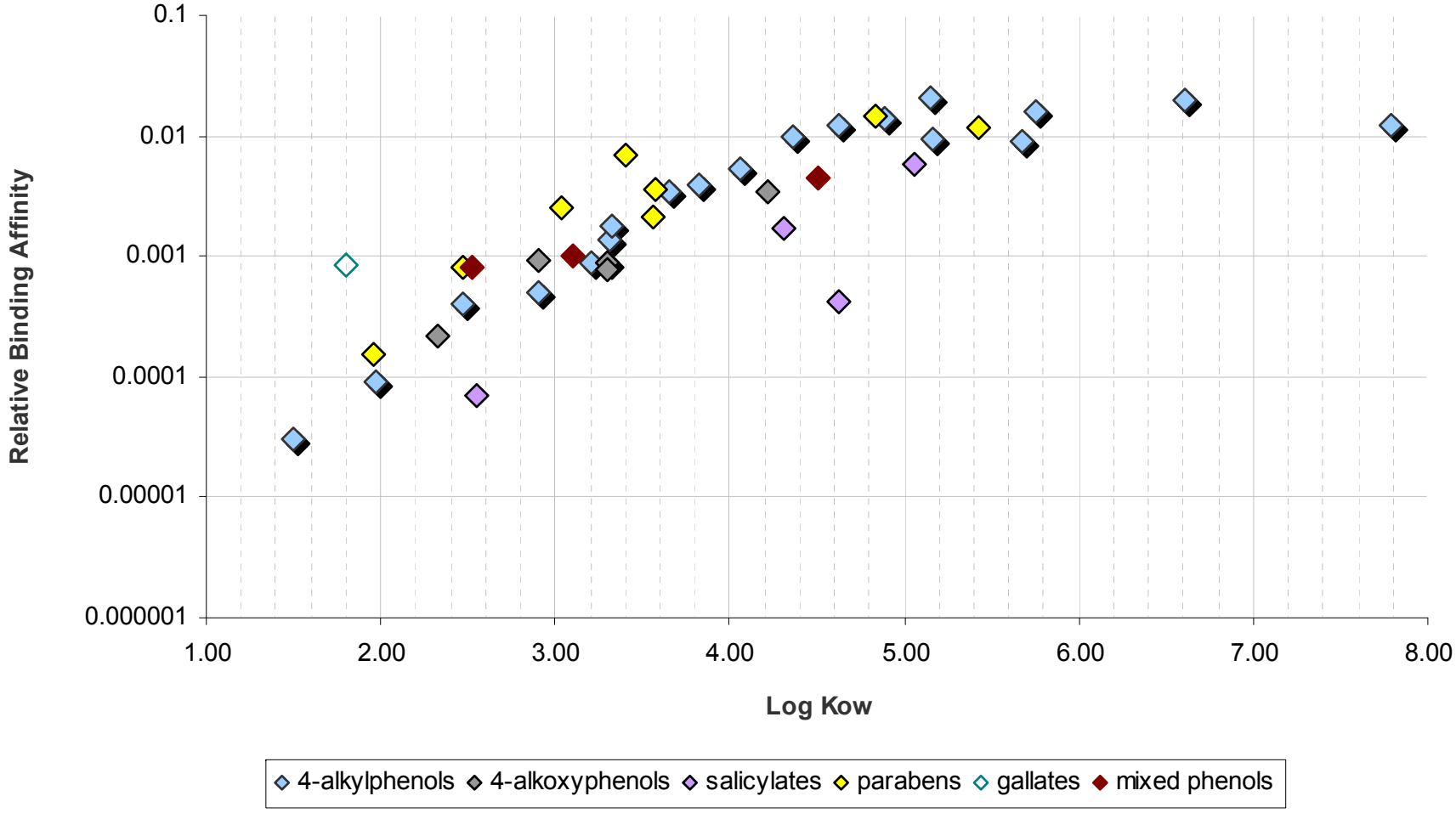


Relationship between Log Kow and *in vitro* relative binding affinity (RBA) for *p*-alkylphenols

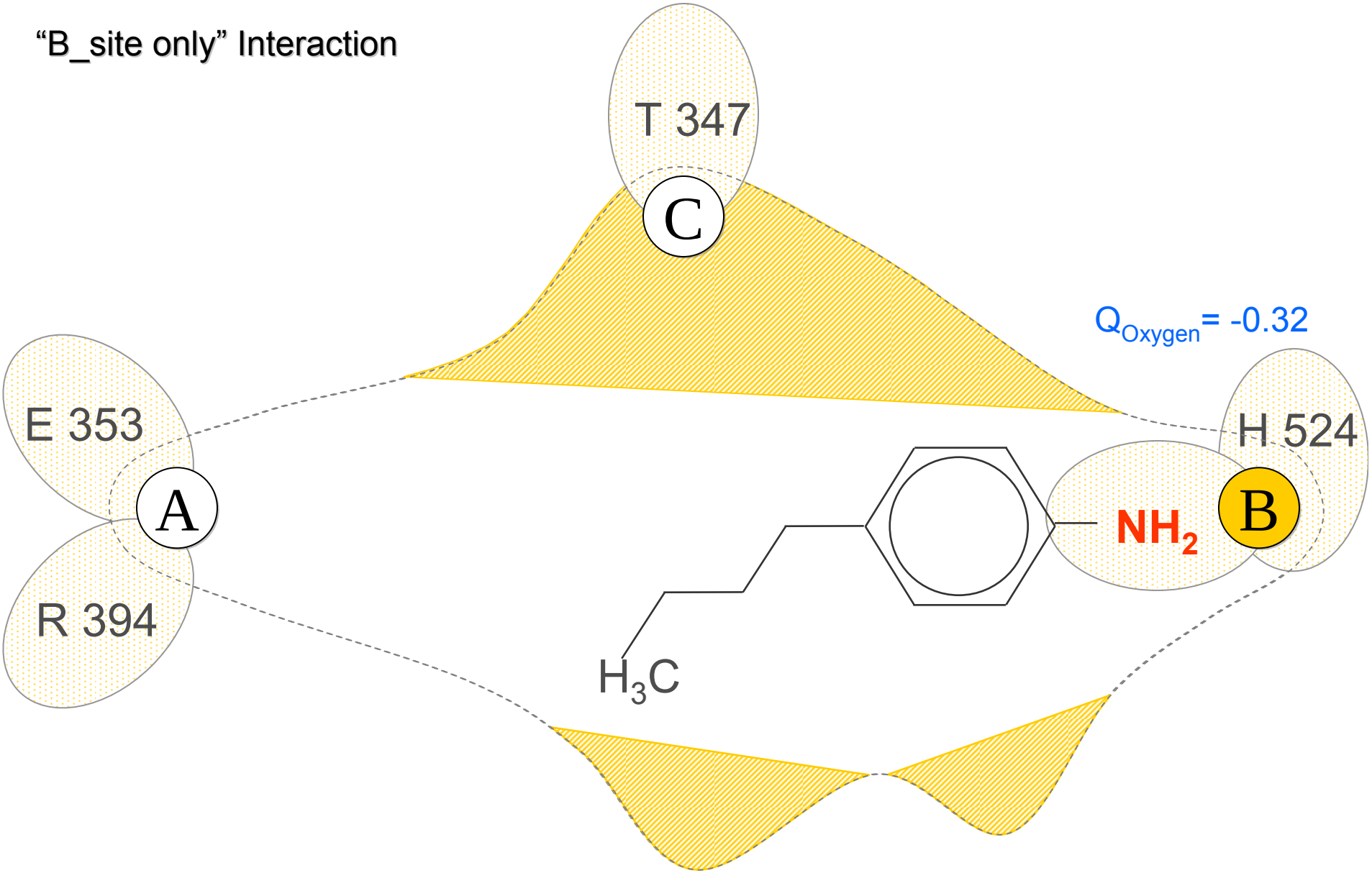


Site A

Chemicals of RBA > 0.00001%



“B_site only” Interaction

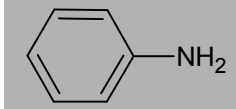
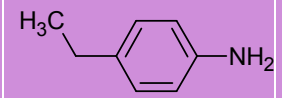
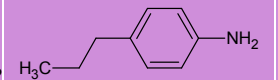
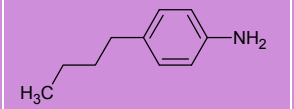
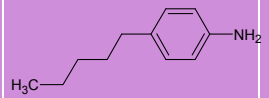
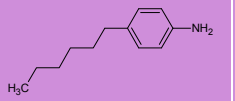
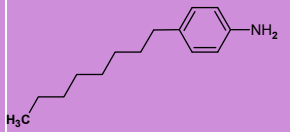


J. Katzenellenbogen

ER Binding **Site B** Homologous Series

4-*n*-Alkylanilines

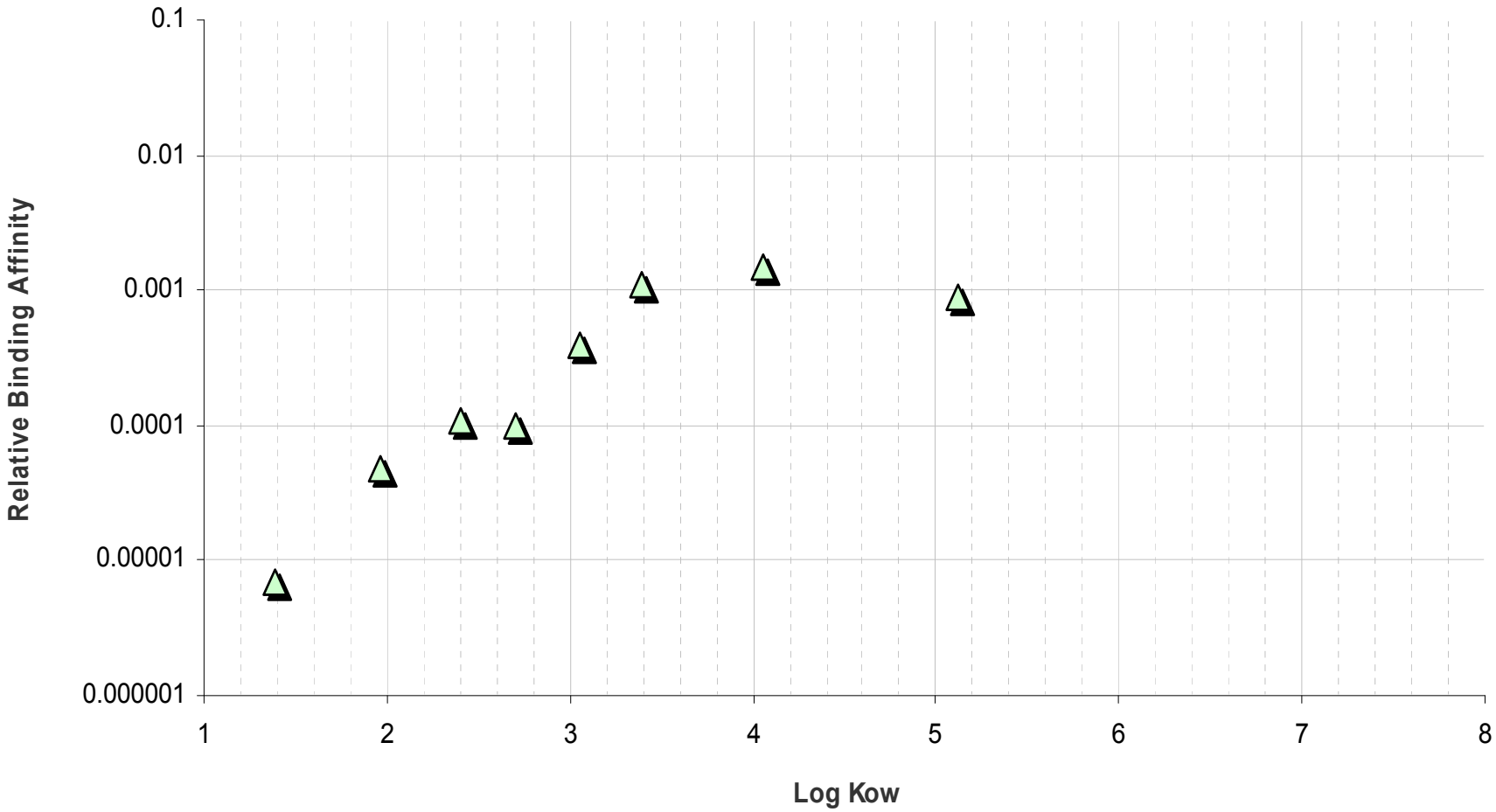
Trout ER Knowledge base

C0	C1	C2	C3	C4	C5	C6	C8
							
Log Kow = 0.90	Log Kow = 1.39	Log Kow = 1.96	Log Kow = 2.40	Log Kow = 3.05	Log Kow = 3.39	Log Kow = 4.06	Log Kow = 5.12

Note: pink shading indicates RBA > 0.00001%; grey shading indicates RBA < 0.00001%

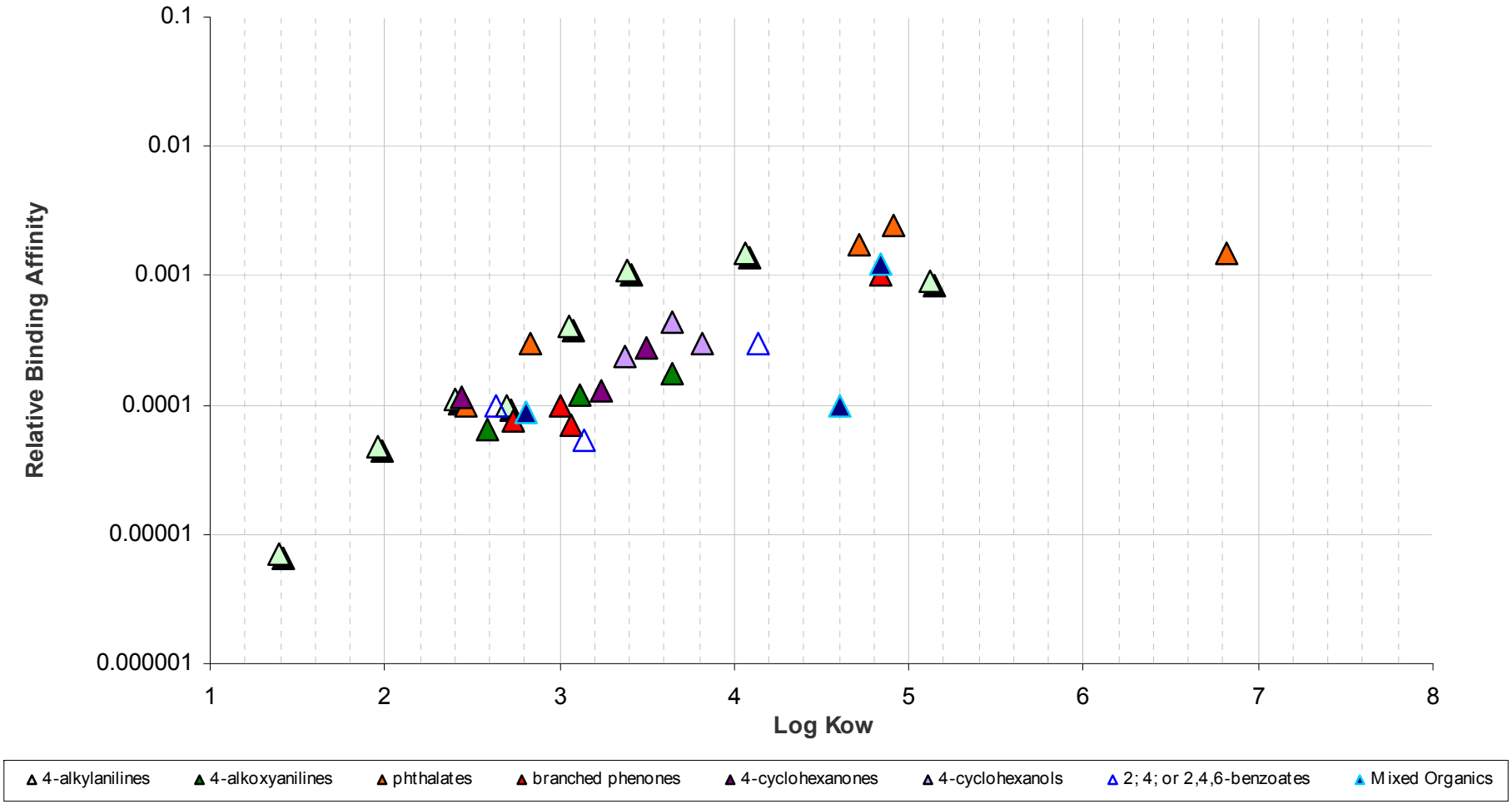
Site B

Relationship between Log Kow and RBA for 4-alkylanilines

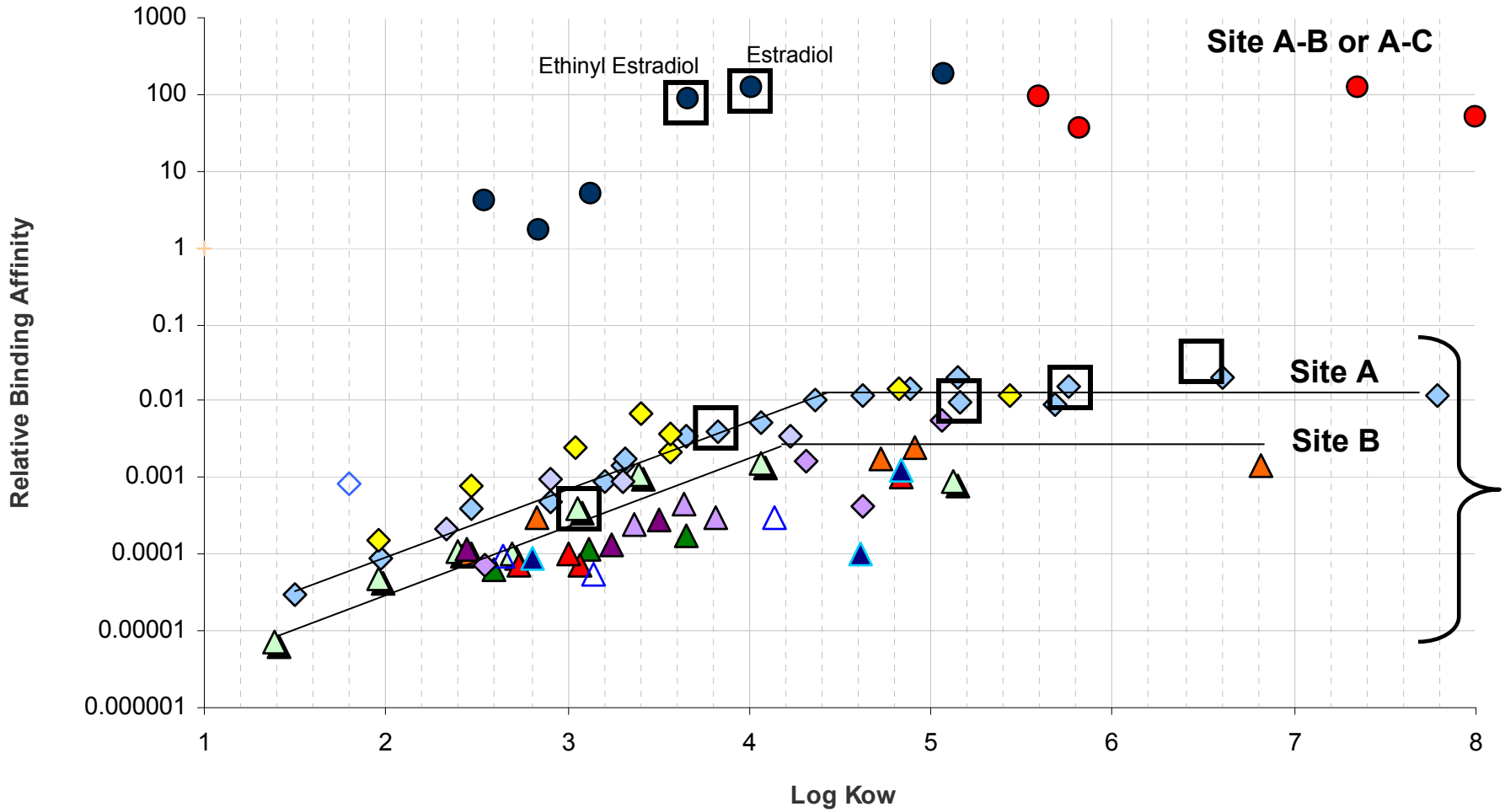


Site B

Chemicals with RBA > 0.00001%

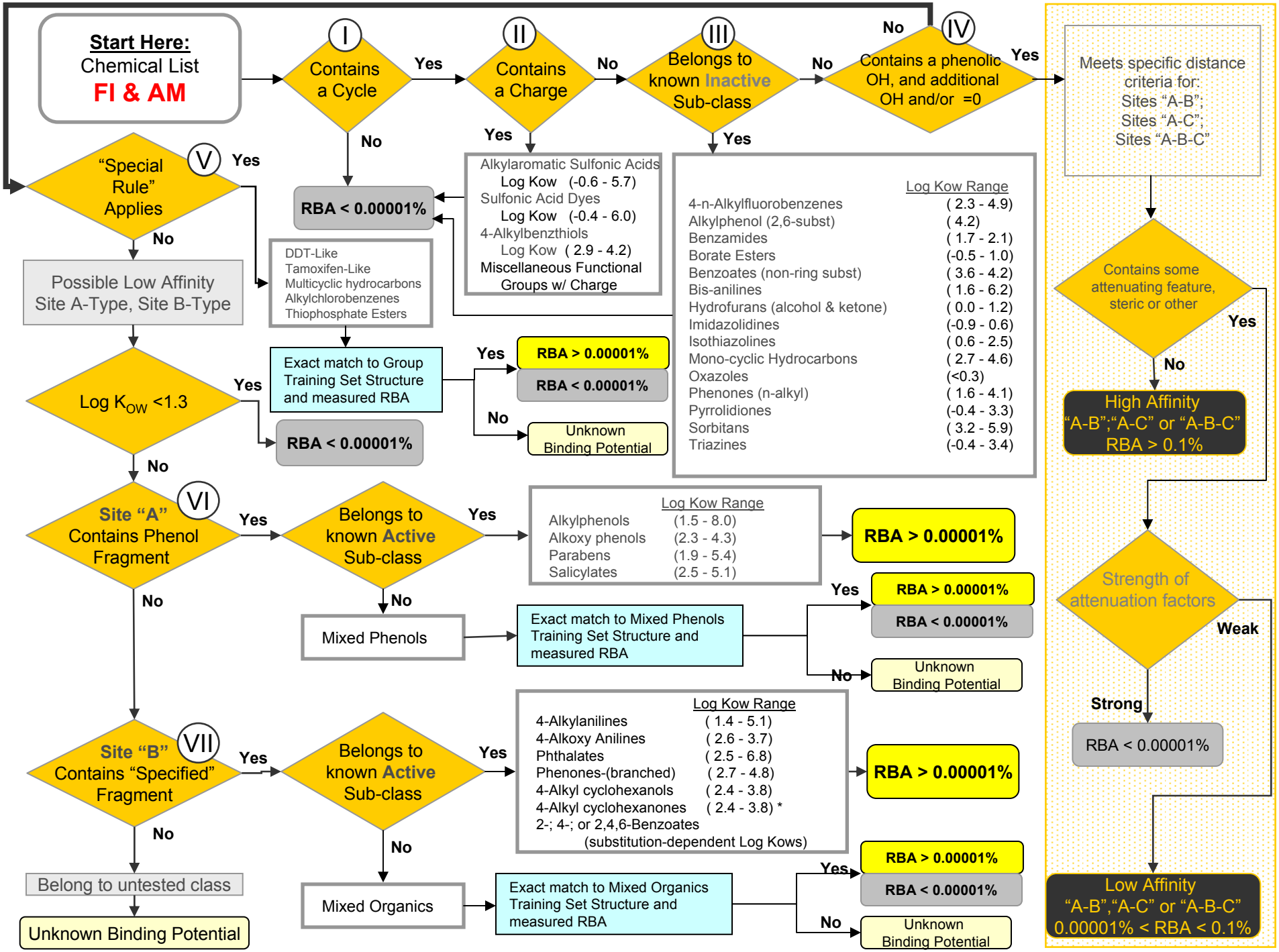


RBAs of low ER affinity chemicals relative to high affinity ER angonists & antagonists



= *in vivo* effects

Note: Blue circles = high affinity ER agonists; orange circles = high affinity ER antagonists


Start Here:
Chemical List
FI & AM
I
Contains a Cycle
Yes
No
II
Contains a Charge
No
Yes
III
Belongs to known Inactive Sub-class
No
Yes
IV
Contains a phenolic OH, and additional OH and/or =0
No
Yes
Meets specific distance criteria for:
Sites "A-B";
Sites "A-C";
Sites "A-B-C"
V
"Special Rule" Applies
Yes
No
RBA < 0.00001%
Alkylaromatic Sulfonic Acids
Log Kow (-0.6 - 5.7)
Sulfonic Acid Dyes
Log Kow (-0.4 - 6.0)
4-Alkylbenzthiols
Log Kow (2.9 - 4.2)
Miscellaneous Functional Groups w/ Charge
Log Kow Range
4-n-Alkylfluorobenzenes (2.3 - 4.9)
Alkylphenol (2,6-subst) (4.2)
Benzamides (1.7 - 2.1)
Borate Esters (-0.5 - 1.0)
Benzoates (non-ring subst) (3.6 - 4.2)
Bis-anilines (1.6 - 6.2)
Hydrofurans (alcohol & ketone) (0.0 - 1.2)
Imidazolidines (-0.9 - 0.6)
Isothiazolines (0.6 - 2.5)
Mono-cyclic Hydrocarbons (2.7 - 4.6)
Oxazoles (<0.3)
Phenones (n-alkyl) (1.6 - 4.1)
Pyrrolidiones (-0.4 - 3.3)
Sorbitans (3.2 - 5.9)
Triazines (-0.4 - 3.4)
Possible Low Affinity Site A-Type, Site B-Type
DDT-Like
Tamoxifen-Like
Multicyclic hydrocarbons
Alkylchlorobenzenes
Thiophosphate Esters
Exact match to Group Training Set Structure and measured RBA
Yes
RBA > 0.00001%
RBA < 0.00001%
No
Unknown Binding Potential
Log K_{OW} <1.3
Yes
RBA < 0.00001%
No
Contains some attenuating feature, steric or other
Yes
No
High Affinity
"A-B";"A-C" or "A-B-C"
RBA > 0.1%
VI
Site "A" Contains Phenol Fragment
Yes
No
Belongs to known Active Sub-class
Yes
No
Log Kow Range
Alkylphenols (1.5 - 8.0)
Alkoxy phenols (2.3 - 4.3)
Parabens (1.9 - 5.4)
Salicylates (2.5 - 5.1)
RBA > 0.00001%
Mixed Phenols
Exact match to Mixed Phenols Training Set Structure and measured RBA
Yes
RBA > 0.00001%
RBA < 0.00001%
No
Unknown Binding Potential
Strength of attenuation factors
Weak
Strong
RBA < 0.00001%
VII
Site "B" Contains "Specified" Fragment
Yes
No
Belongs to known Active Sub-class
Yes
No
Log Kow Range
4-Alkylanilines (1.4 - 5.1)
4-Alkoxy Anilines (2.6 - 3.7)
Phthalates (2.5 - 6.8)
Phenones-(branched) (2.7 - 4.8)
4-Alkyl cyclohexanols (2.4 - 3.8)
4-Alkyl cyclohexanones (2.4 - 3.8) *
2-; 4-; or 2,4,6-Benzoates
(substitution-dependent Log Kows)
RBA > 0.00001%
Belong to untested class
Unknown Binding Potential
Mixed Organics
Exact match to Mixed Organics Training Set Structure and measured RBA
Yes
RBA > 0.00001%
RBA < 0.00001%
No
Unknown Binding Potential
Low Affinity
"A-B","A-C" or "A-B-C"
0.00001% < RBA < 0.1%

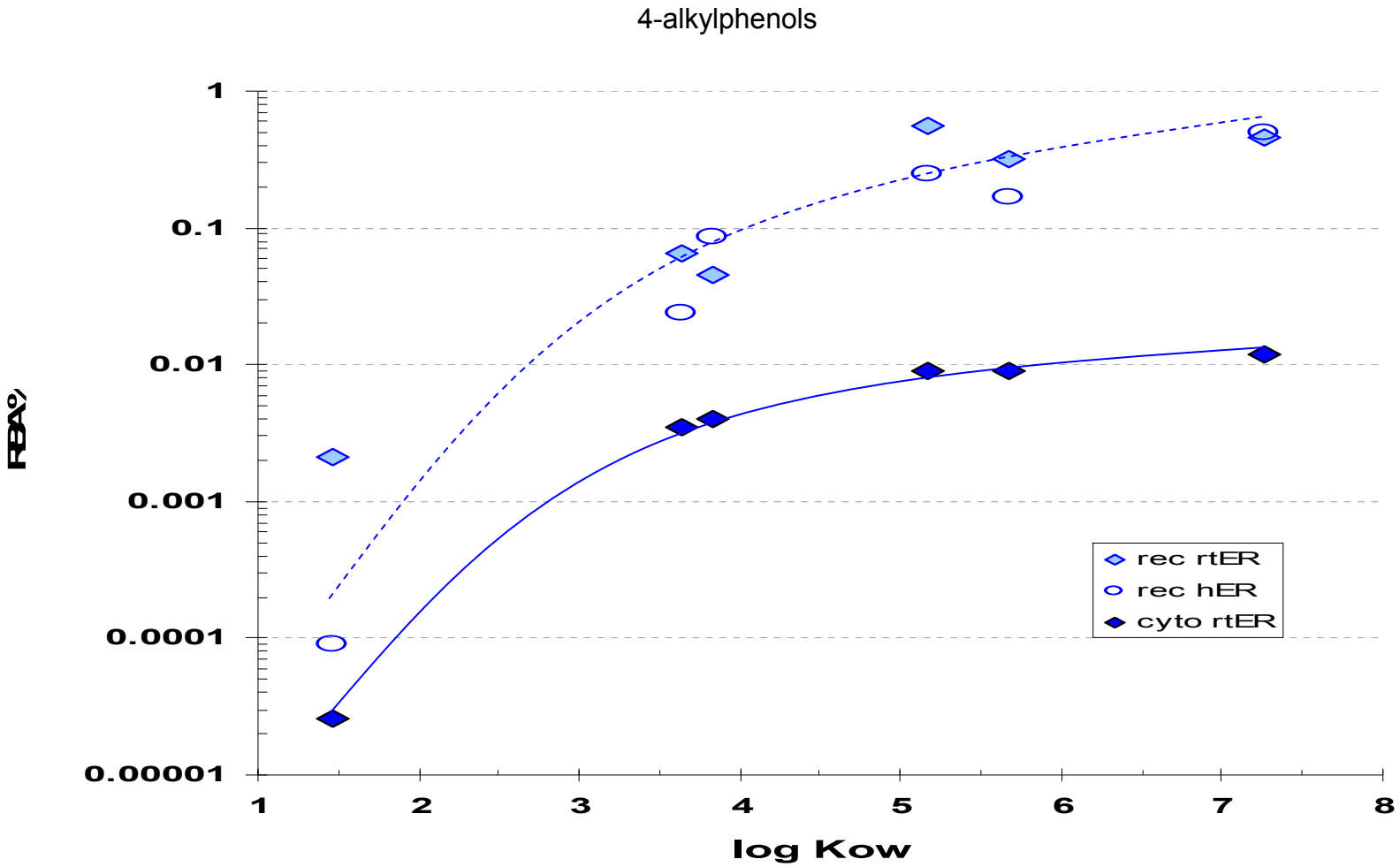
Expert System Predictions for Food use Inerts and Antimicrobials

	Food use Inerts	*Antimicrobials*
	Total Chemicals (%)	*Total Chemicals (%)*
	393 (100%)	211 (100%)
Predicted RBA < 0.00001	378 (96%)	196 (93%)
Predicted RBA > 0.00001	15 (4%)	15 (7%)

Ongoing Research:

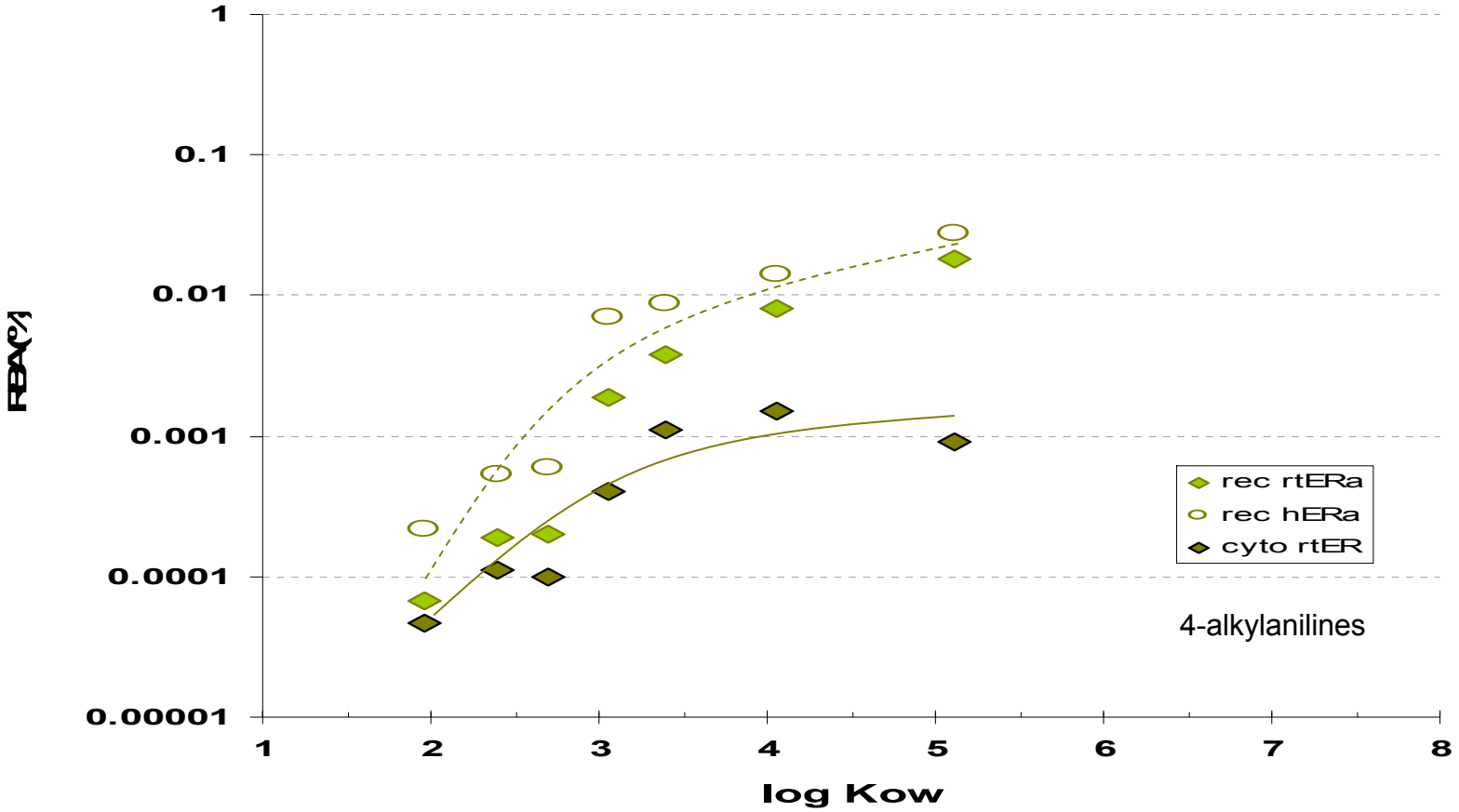
Human ER Binding Affinity and Gene Activation

4-alkylphenols (**Site A)**
human ER vs. trout ER



RBA more comparable when assay chemical bioavailability is similar regardless of species (rec hERα and rec rtERα) vs (cyto rtER).

4-alkylanilines (**Site B)**
human ER vs. trout ER



RBA more comparable when assay chemical bioavailability is similar regardless of species (rec hERα vs. rec rtERα) vs. (cyto rtER)

Highlights

- Strategic Testing and QSAR-based model development to cover specific chemical inventories of regulatory concern
  - Regulatory Domain; Assay Domain; Model Domain

- Chemical concentrations tested in assays are based on chemical behavior in *in vitro* assays
  - Cell-free assay – test for effect up to solubility limit
  - Cell-based assay – test for effect up to solubility or toxicity, whichever comes first

- Bioavailability