Update on Research Using *in vitro* and Computer-based Tools for Screening Potential Estrogenic Activity

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Quantitative Structure-Activity Relationships Assumptions

- A chemical's structure imparts properties
- A group of chemicals that produce the same biological activity (toxicity; adverse effect) have something similar about their chemistry (structure)
- Goal is to quantify 'structural similarity' imparting biological activity; identify which other chemicals may be 'similar' with the assumption that an untested chemical may produce the same activity

Chemical similarity is defined in the context of biological similarity

- Robustness Depends on:
  - Well-defined biological system; Well-characterized chemistry
  - Well-defined application –
    - Risk context - What's the question being asked - problem definition

QSAR Assumption

Chemical Structure/ Property ⟷ Toxicological Potential

Δ Chemical Structure/ Property → Δ Dose Metric (kinetics/ metabolism) → Δ Endpoint Potency

Toxic potency is correlated to chemical concentration at the site of action

-C. Hansch

Well-defined system (chemistry and biology)

Well-Defined Biological System
(What do you know and what are you assuming)

- Is the chemical administered what you thought it was
  - Impurities
- Metabolism
  - Is the system used for collection of empirical data capable of xenobiotic metabolism?
  - Is what you're measuring due to parent chemical or to a metabolite?
- Kinetics
  - What do you understand about the chemical kinetics within the system?
  - Is the chemical in solution
    - Bound and unavailable
    - Loss to hydrolysis

Has chemical form and/or concentration been <u>measured</u> in the biological system upon which the QSAR is based

QSAR Approach

- QSAR is approach to help think about, hypothesize, and investigate, in a systematic manner how a chemical is most likely to interact with a biological system and what adverse effect might be the consequence of that interaction
- QSAR depends upon a well-defined biological system
- QSAR for large diverse chemical inventories is an Iterative process
- How QSAR used depends upon the regulatory context
  - Defining the regulatory domain is non-trivial; identify the exact chemicals and verify structures
  - Defining the regulatory question is essential; regulatory acceptance criteria are dependent upon the use

Risk Context

Development and use of a QSAR in regulatory risk assessment requires clear problem definition

- The purpose of the QSAR application must be well-defined (e.g., priority setting for testing, and chemical-specific risk assessment are two very different purposes – different acceptance criteria)

- The chemicals of regulatory concern must be defined to establish an appropriate training set for QSAR development and/or to assess appropriateness of QSAR application
  - Regulatory Domain
  - Applicability Domain of QSAR (dependent on Training Set)

A QSAR can only be as good as the underlying toxicological understanding and data it is based upon

- Toxicological activity is assessed based on a well-defined endpoint in a well-defined assay
  - e.g., chemical dosimetry –
  - if you assume parent chemical is responsible for biological activity but in fact a metabolite produced toxicity, then you're working from wrong structure
  - If you assume chemical was 100% available in your system but in fact 80% was loss due to volatility, or binding to glassware, unavailable in vehicle administered, etc then your concentration may have to be corrected

Today's Research Update: Developing the Tools to move EPA toward the New Paradigm

- Use screening and priority setting to focus on the most plausible toxicological potential for chemical or group of chemicals, not all possible adverse outcomes.

- Challenge of implementing FQPA
  - Endocrine Disruptors - How to prioritize and efficiently test a large number of chemicals while still carrying out existing chemical (new and old) evaluation programs

- Hypothesis-driven approach

QSARs for Prioritization

Food Quality Protection Act –
Need to prioritize *in vivo* testing options for classes of compounds where 'endocrine data' is lacking:

- •Inert ingredients used in formulations of pesticides used on crops
- •Antimicrobial active ingredient pesticides

Prioritize -

- •Based on effect endpoint(s) in combination with existing exposure estimates
- • Use QSARs to estimate potential for 'estrogenic activity' for untested inerts and antimicrobial pesticides

Research Focus:

- Adverse outcome pathway:
  - Reproductive impairment through the ER-mediated pathway
- Chemicals:
  - Inert ingredients
  - Antimicrobials
- Hypothesis-driven approach:
  - Chemicals that have similar activity will have similar structure; quantifying the structural similarity will allow extrapolation across chemicals

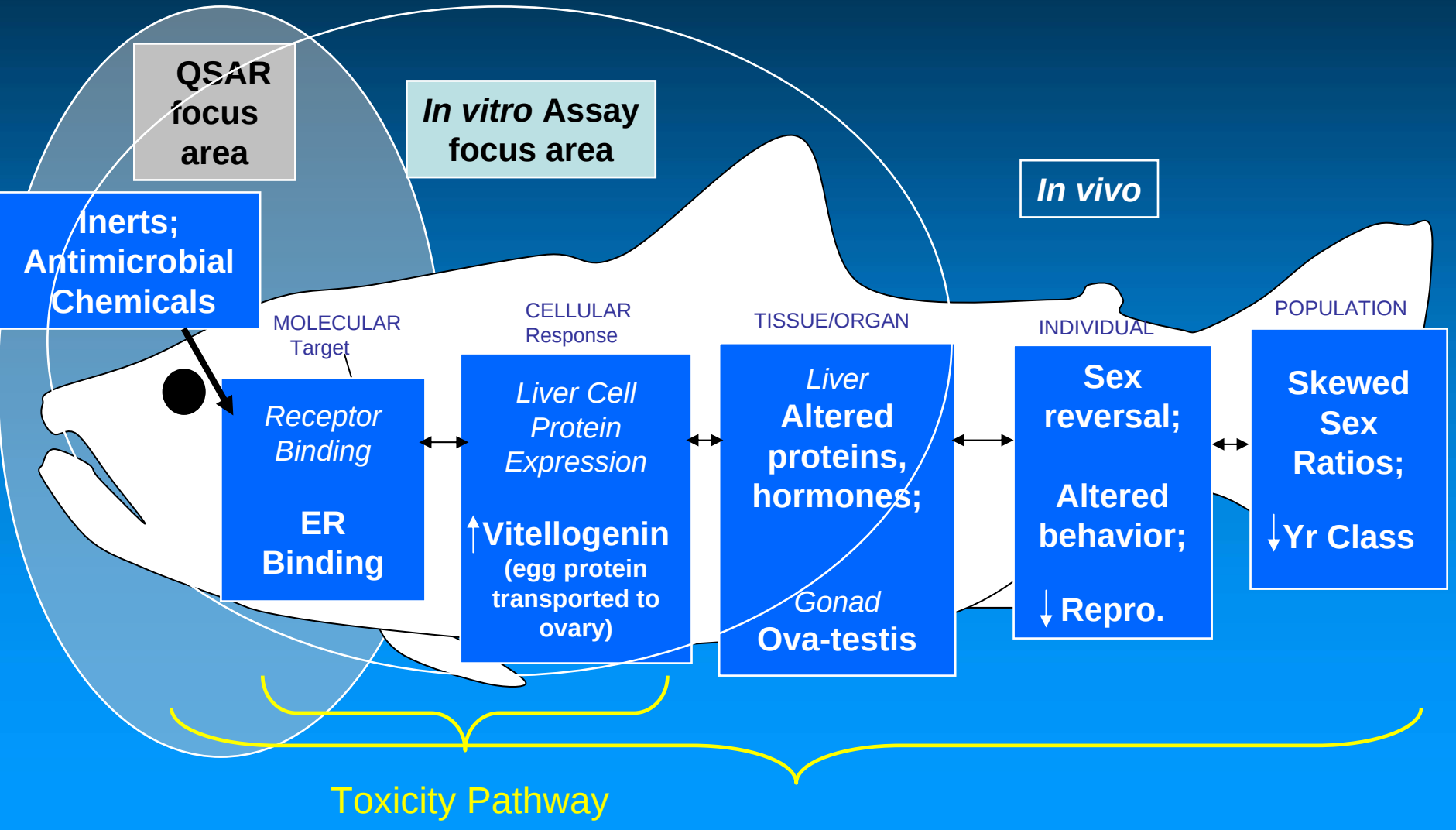
Research Approach:

- Test a ‘representative’ chemicals *in vitro* to extrapolated potential for activity to untested

- Chemical Class Approach based on mechanism:
  - What types of chemicals can interact with the ER and which ones can’t

- *in vitro* assays:
  - ER binding displacement
  - ER-mediated gene activation

Adverse Outcome Pathway
ER-mediated Reproductive Impairment
Measurements made across levels of biological organization



Defining the Problem:
Prioritizing estrogenic potential of Food Use Inert Ingredients

Inert chemicals in Pesticides used on Food Crops
The 2004 List included:

893 entries = 393 discrete chemicals + 500 non-discrete substances
(44% discrete : 56% non-discrete)

393 discrete chemicals include:
- **366 organics (93%)**
- **24 inorganics (6%)**
- **3 organometallics (1%)**

500 non-discrete substances include:
- **147 polymers of mixed chain length**
- **170 mixtures**
- **183 undefined substances**

Defining the Problem: *Prioritizing Estrogenic Potential of Antimicrobial Pesticides*

Antimicrobials and Sanitizers
List included:

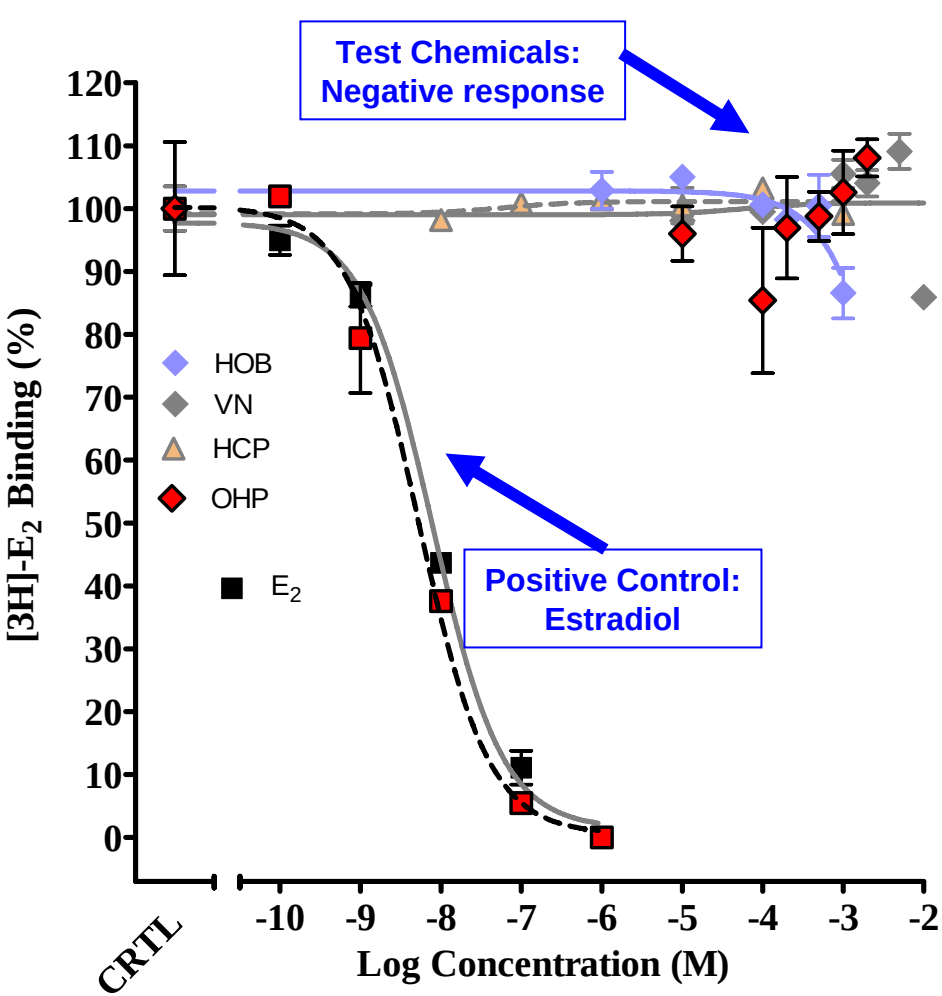
299 = 211 discrete chemicals + 88 non-discrete substances
(71% discrete : 29% non-discrete)

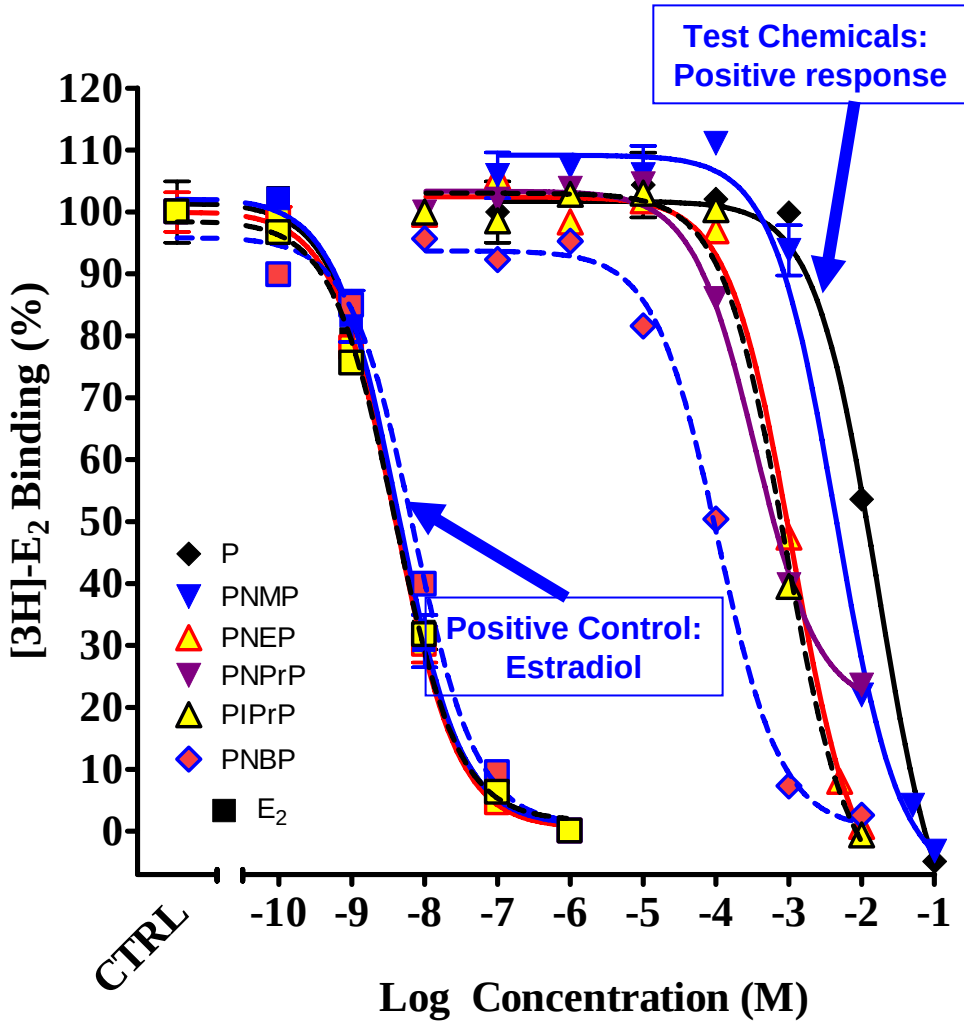
211 discrete chemicals include:
153 organics (72%)
52 inorganics (25%)
6 organometallics-acyclic (3%)

88 non-discrete substances include:
25 polymers of mixed chain length
35 mixtures
28 undefined substances

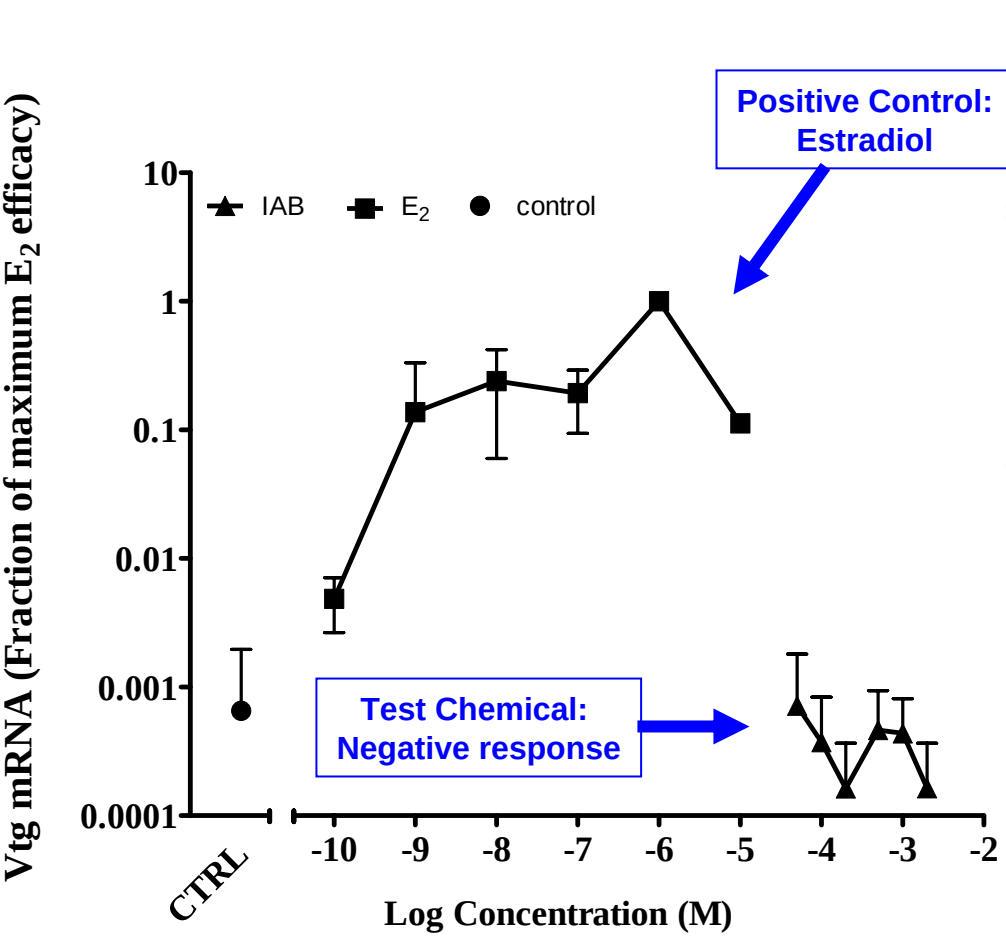
Data Example - primary *In vitro* assay used :

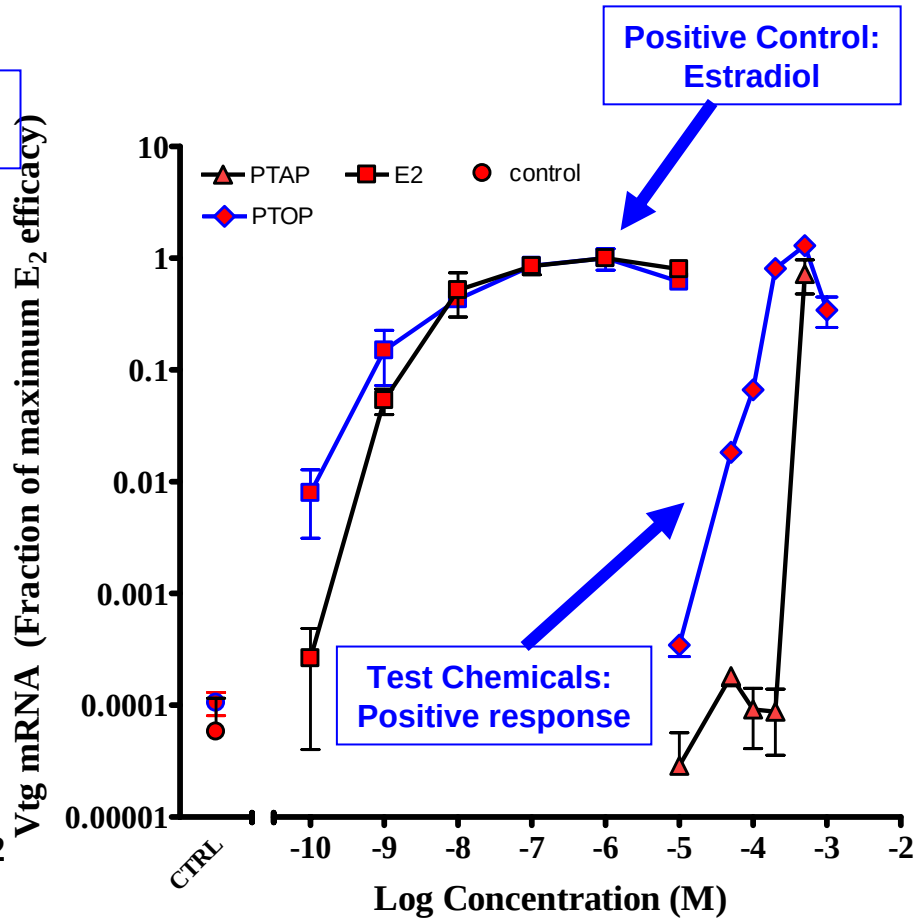
Estrogen Receptor Binding Displacement Assay





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





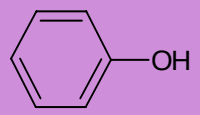
Research Approach:

- Test a few ‘representative’ chemicals *in vitro* to extrapolate to others

- Chemical Class Approach based on mechanism:
  - What types of chemicals can interact with the ER and which ones can’t
    - chemicals selected to investigate mechanisms of binding the ER
    - chemicals selected to cover classes found on list

Homologous Series
Alkylphenols

C0



Log Kow = 1.50 $_{msrd}$

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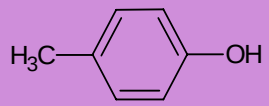

1.97 msrd

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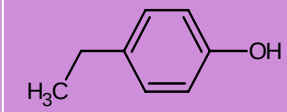

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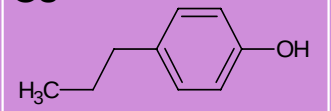

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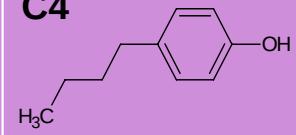

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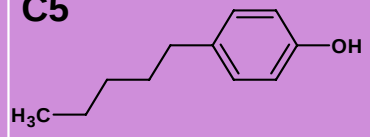

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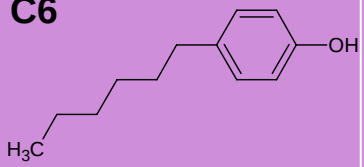

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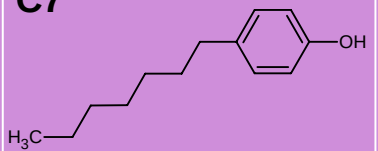
C7



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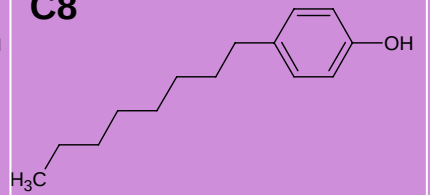

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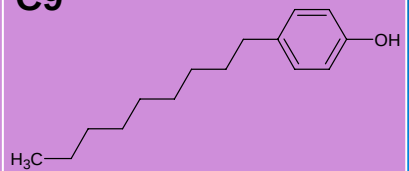
C9



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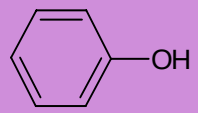
Alkylphenols

C0



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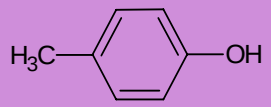

1.97 msrd

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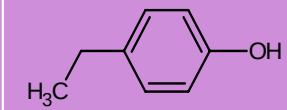

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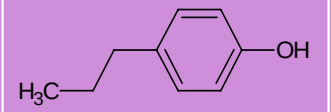

3.20 msrd

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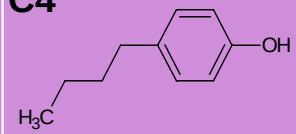

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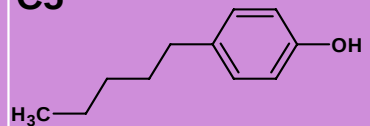

4.06 msrd

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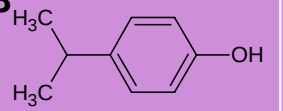

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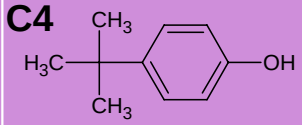

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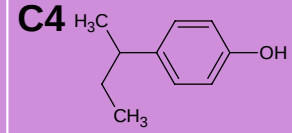

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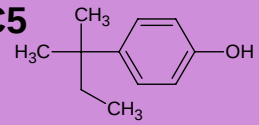

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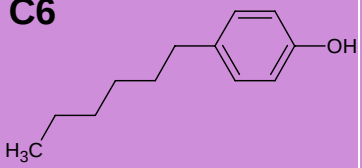

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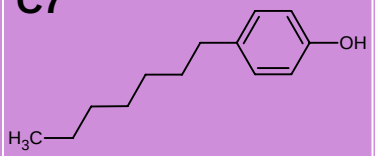

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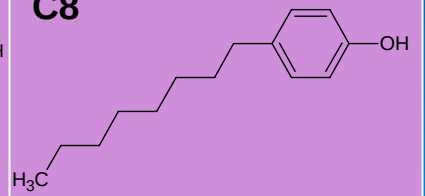

5.68 calc

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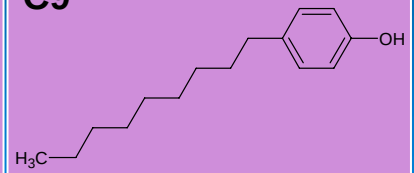

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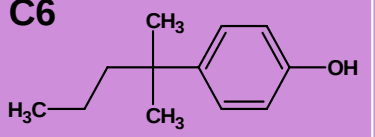

4.36 clog

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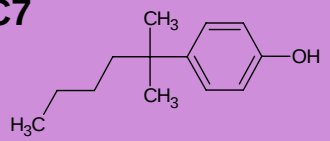

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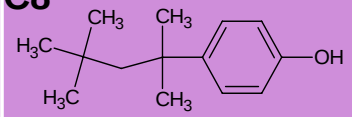

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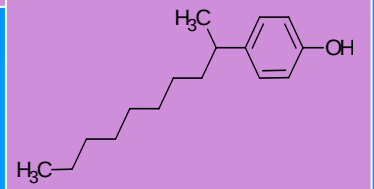

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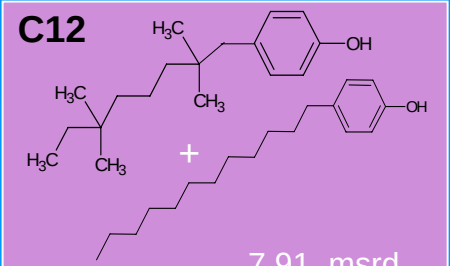

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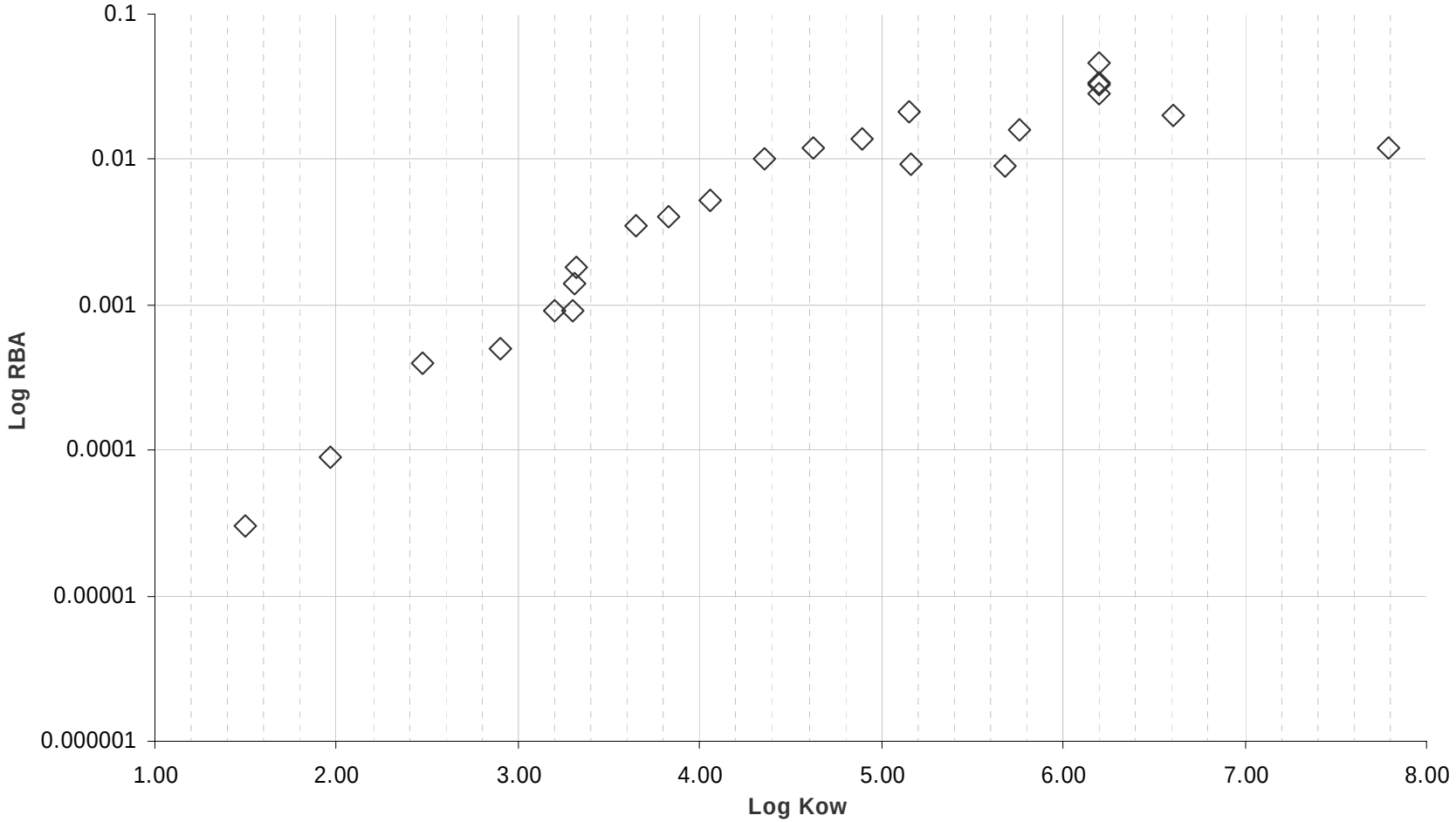
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1.00
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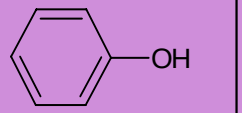
7.00
8.00
Log Kow

Alkylphenols – (p-branched chain)

rtER tested chemicals - Training Set

C0



Log Kow = 1.50 msrd

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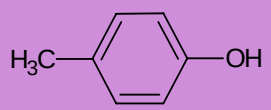

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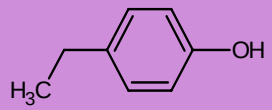

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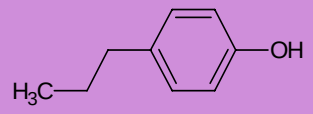

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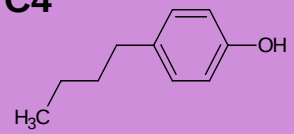

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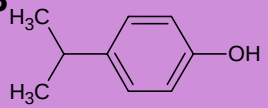

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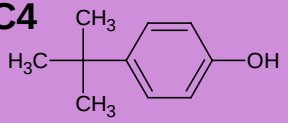

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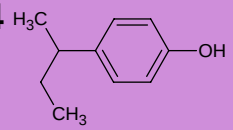

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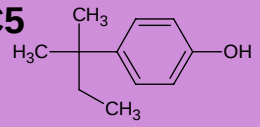

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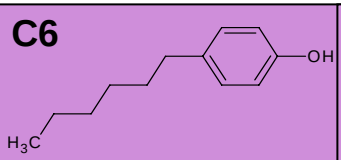
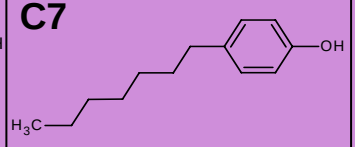


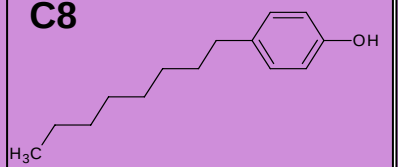
4.62 calc

C7



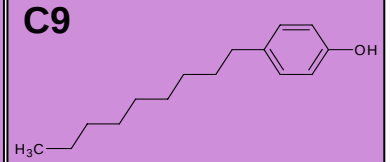
4.15 msrd

C8



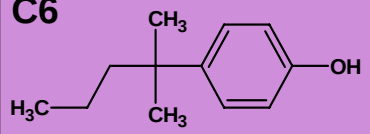
5.68 calc

C9



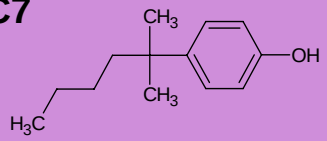
5.76 msrd

C6



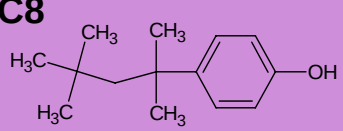
4.36 clog

C7



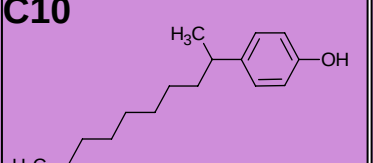
4.89 clog

C8



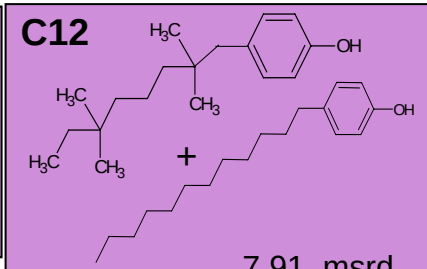
5.16 clog

C10



6.61 clog

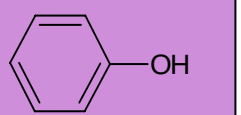
C12



7.91 msrd

Inventory

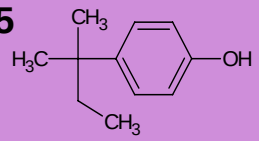
C0



Log Kow = 1.50 msrd

Inventory

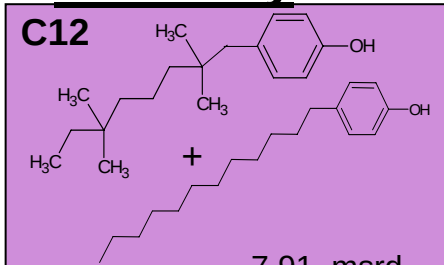
C5



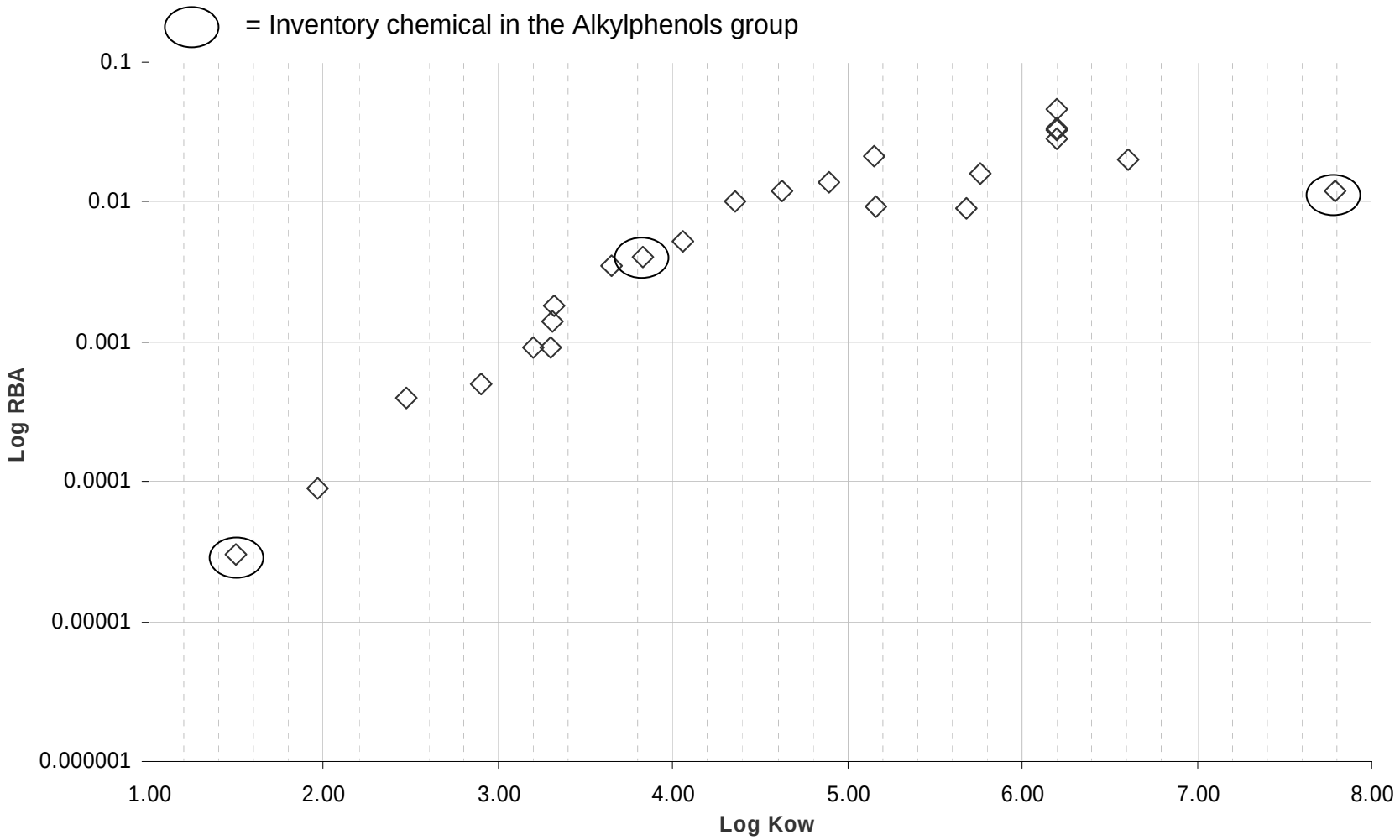
3.83 msrd

Inventory

C12

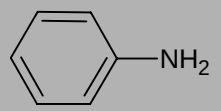


7.91 msrd

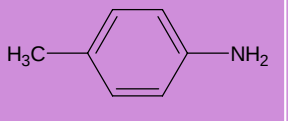

= Inventory chemical in the Alkylphenols group
0.1
0.01
0.001
0.0001
0.00001
0.000001
Log RBA
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Log Kow

Alkylanilines – (p-n chain)

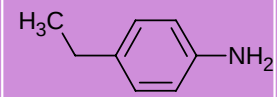
rtER tested chemicals - Training Set



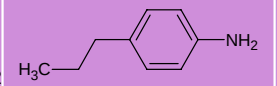
0.90 msrd



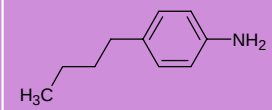
1.39 msrd



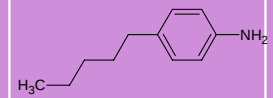
1.96 msrd



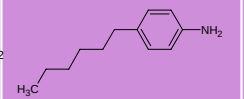
2.40 msrd



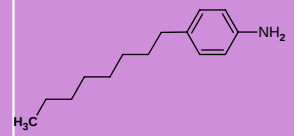
3.05 msrd



3.39 msrd

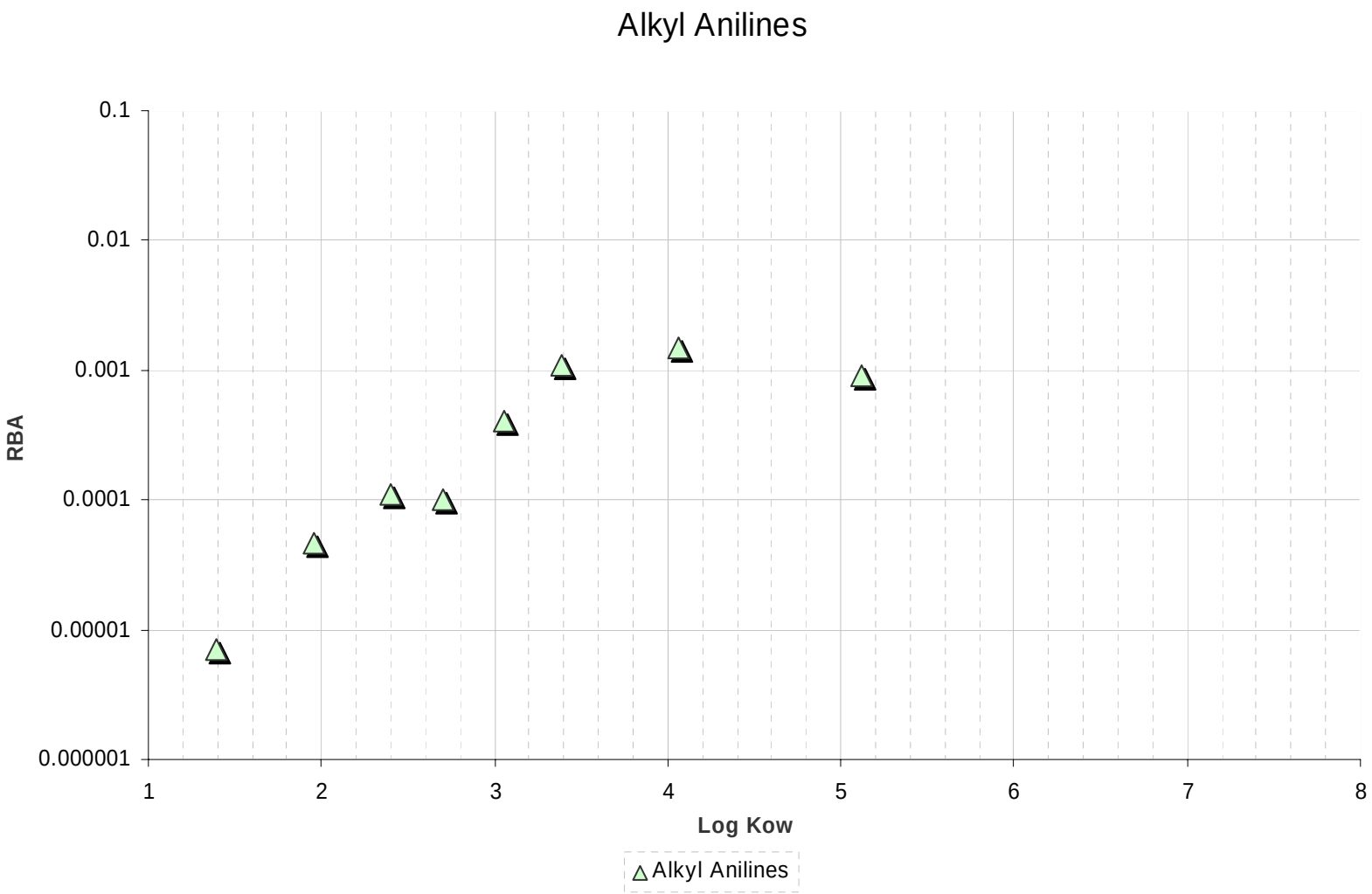


4.06 clog



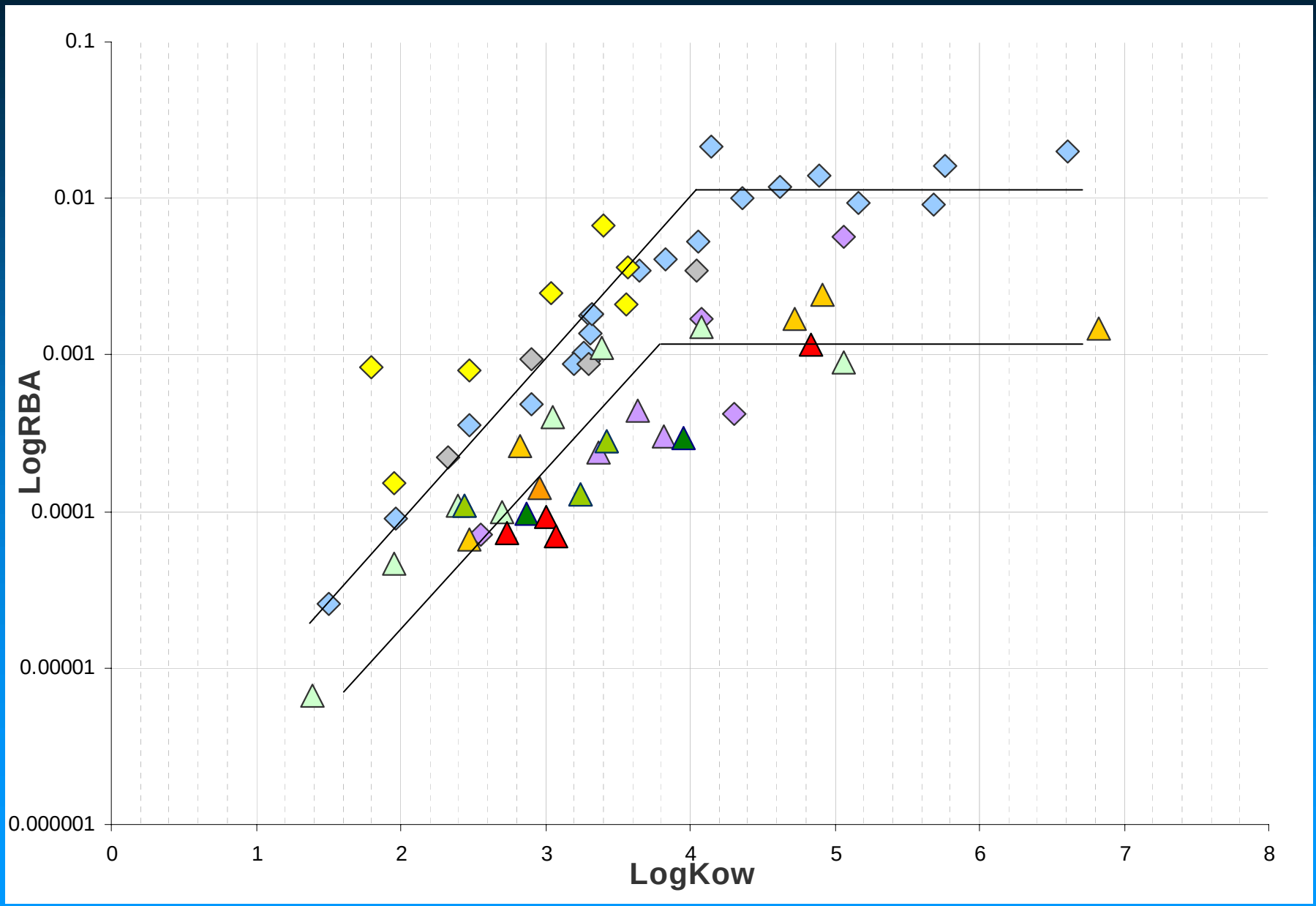
5.12 clog

Figure 5. Relationship between Log Kow and RBA for alkylanilines.

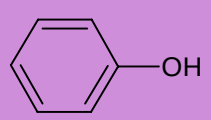
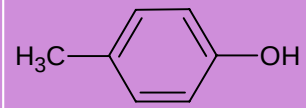
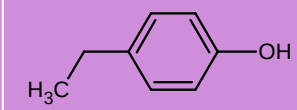
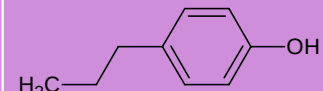
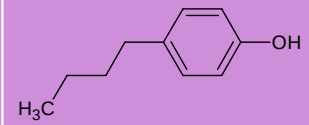
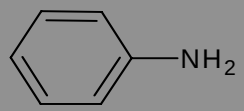
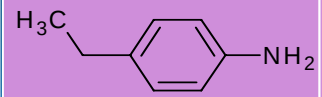
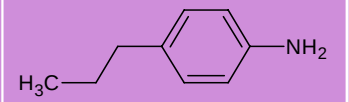
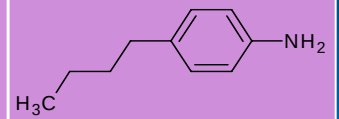
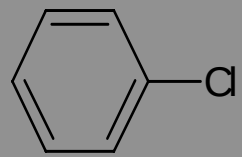
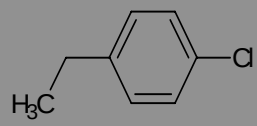
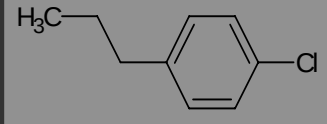
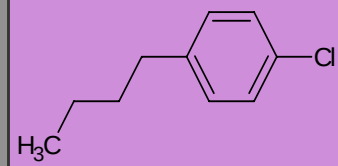
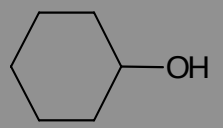
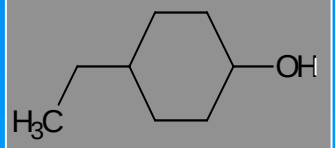
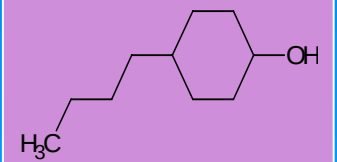


Rainbow Trout ER binding Affinity vs. Log Kow

RBA = relative binding affinity compared to Estradiol at 100%

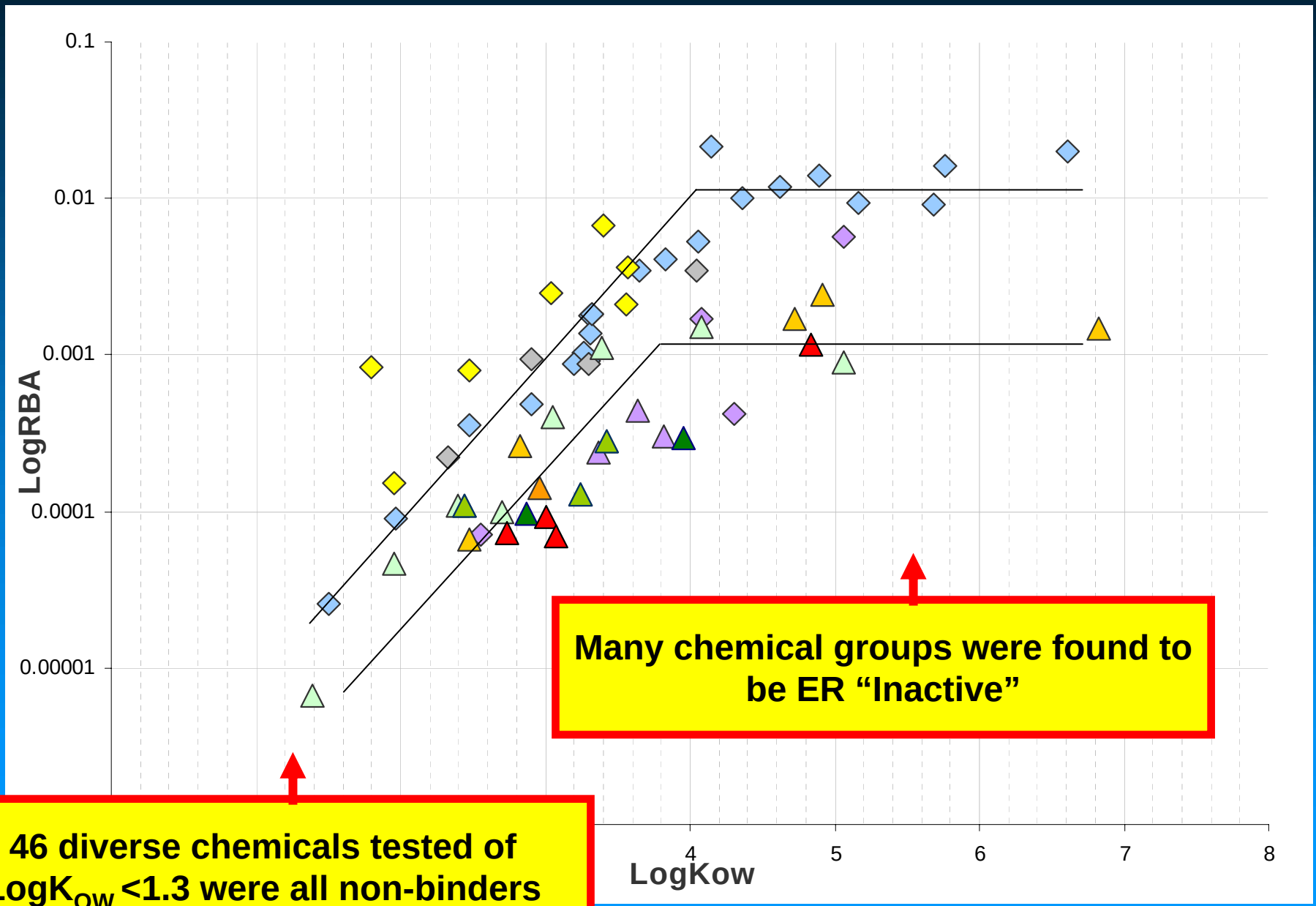


$LogK_{ow}$ Cutoffs vary with Chemical Subgroups

	C0	C1	C2	C3	C4
p,n-Alkyl Phenols	 LogKow=1.50 m	 1.97 m	 2.47 m	 3.20 m	 3.65 m
p,n-Alkyl Anilines	 0.90 m	 1.39 m	 1.96 m	 2.40 m	 3.05 m
p,n-Alkyl Chloro benzenes	 2.84 m	 3.88 c	 4.41 c	 4.94 c	
p,n-Alkyl Cyclo hexanols	 1.23 m	 2.32 c		 3.37 c	

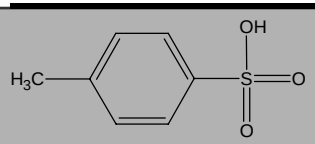
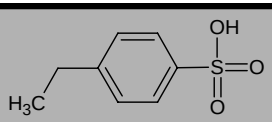
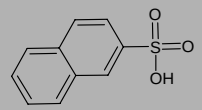
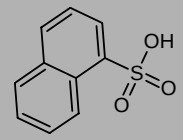
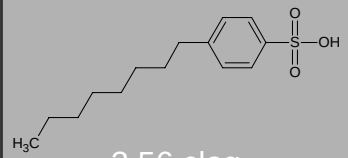
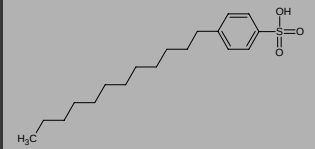
Rainbow Trout ER binding Affinity vs. Log Kow

RBA = relative binding affinity compared to Estradiol at 100%

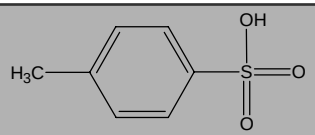
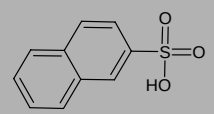
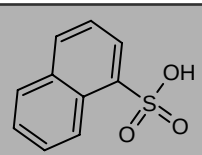
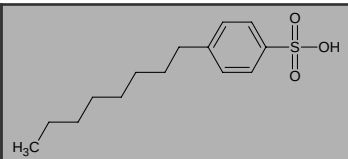
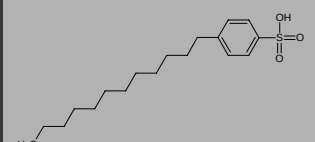


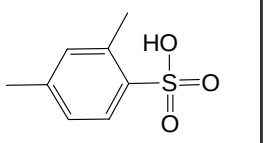
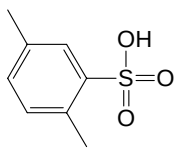
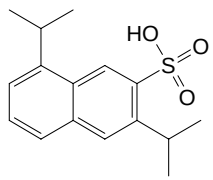
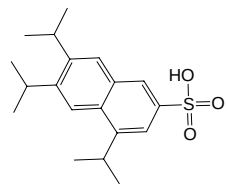
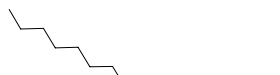
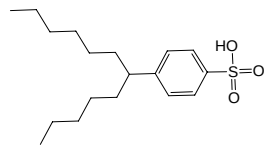
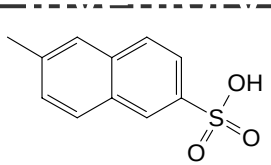
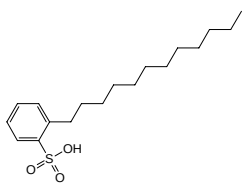
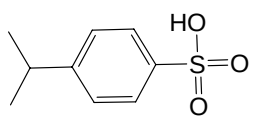
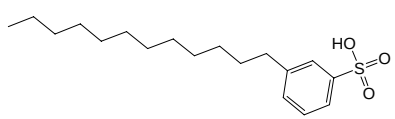
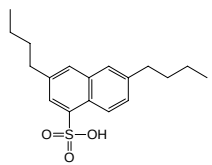
Alkylaromatic sulfonic acids

rtER tested chemicals - Training Set

					
-0.62 msrd	0.38 clog	0.63 msrd	0.92 clog	3.56 clog	5.67 clog

Inventory

					
-0.62 msrd		0.63 msrd	0.92 clog	3.56 clog	5.67 clog

					
					and other variations & salts of structures shown here

Results:

Chemical has Low Potential for Activity if:

-Belongs to a group where testing showed no evidence of ER interaction (RBA < 0.00001);

-LogKow <1.3, or meets other group-specific LogKow cutoffs

General characteristics of these chemicals:

-Acyclic (e.g., no benzene rings)

-Cyclic, but does not contain a likely H-bonding group;

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 (also known as Log P); is an indicator of lipophilicity

Results:

Chemical has Higher Potential for Activity if:

-Belongs to chemical group with evidence of ER interaction, (RBA > 0.00001), and:

-LogKow > 1.3, and < any chemical group-specific high LogKow cutoff

General characteristics of these chemicals:

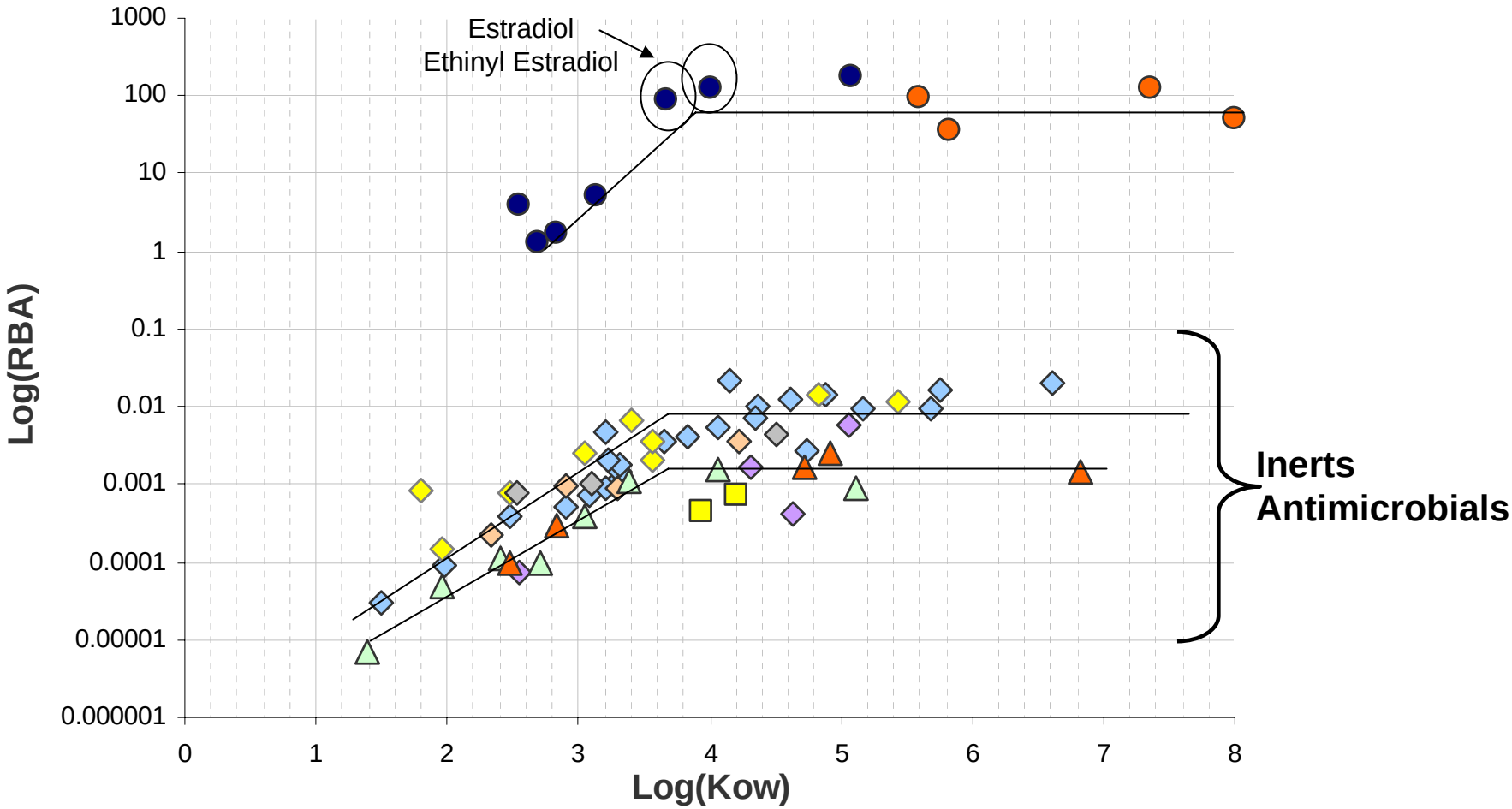
-Contains at least one cycle (e.g., benzene ring);

-Contains a possible H-bonding group;

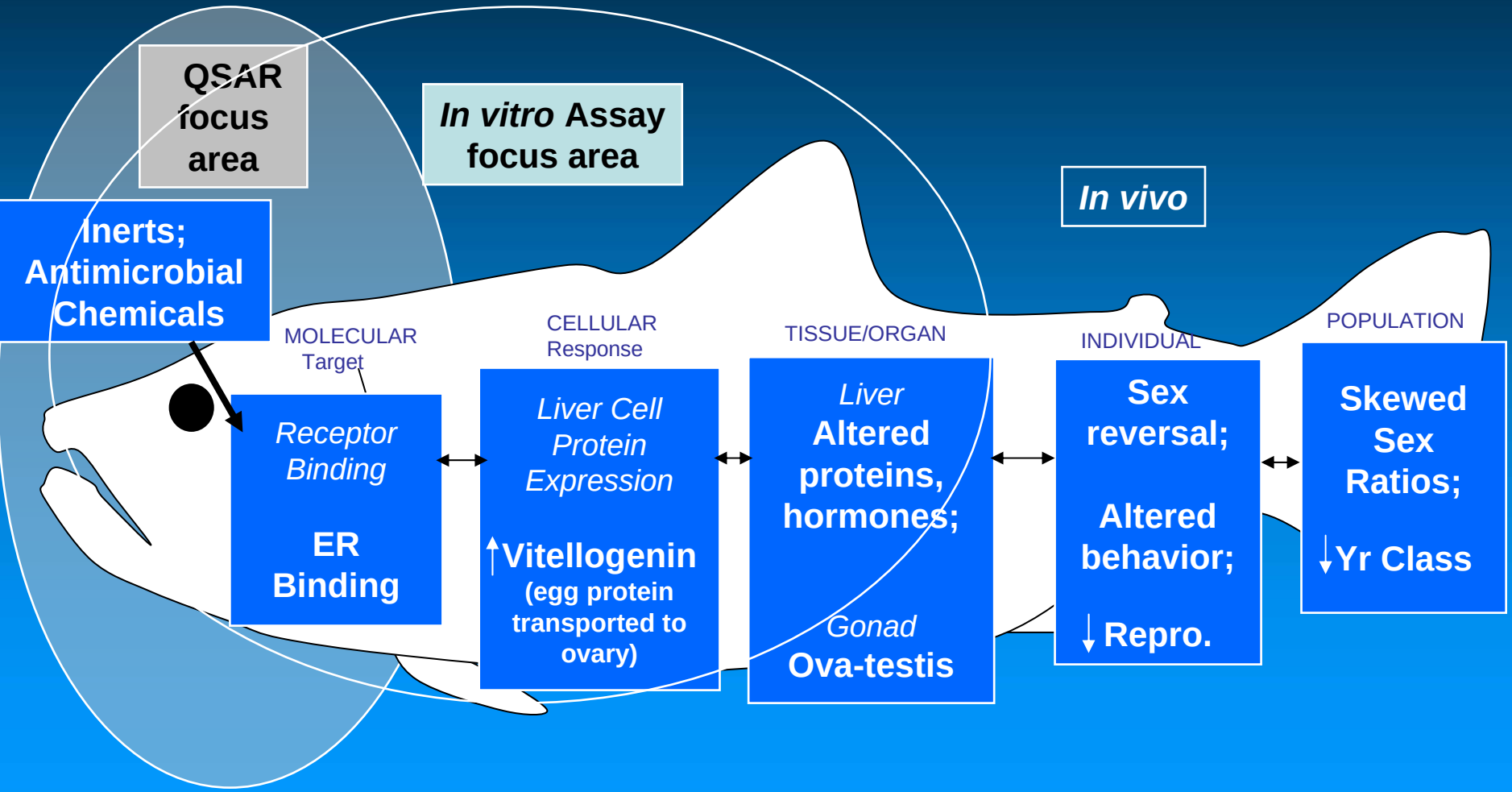
Food Use Inerts, and Antimicrobials

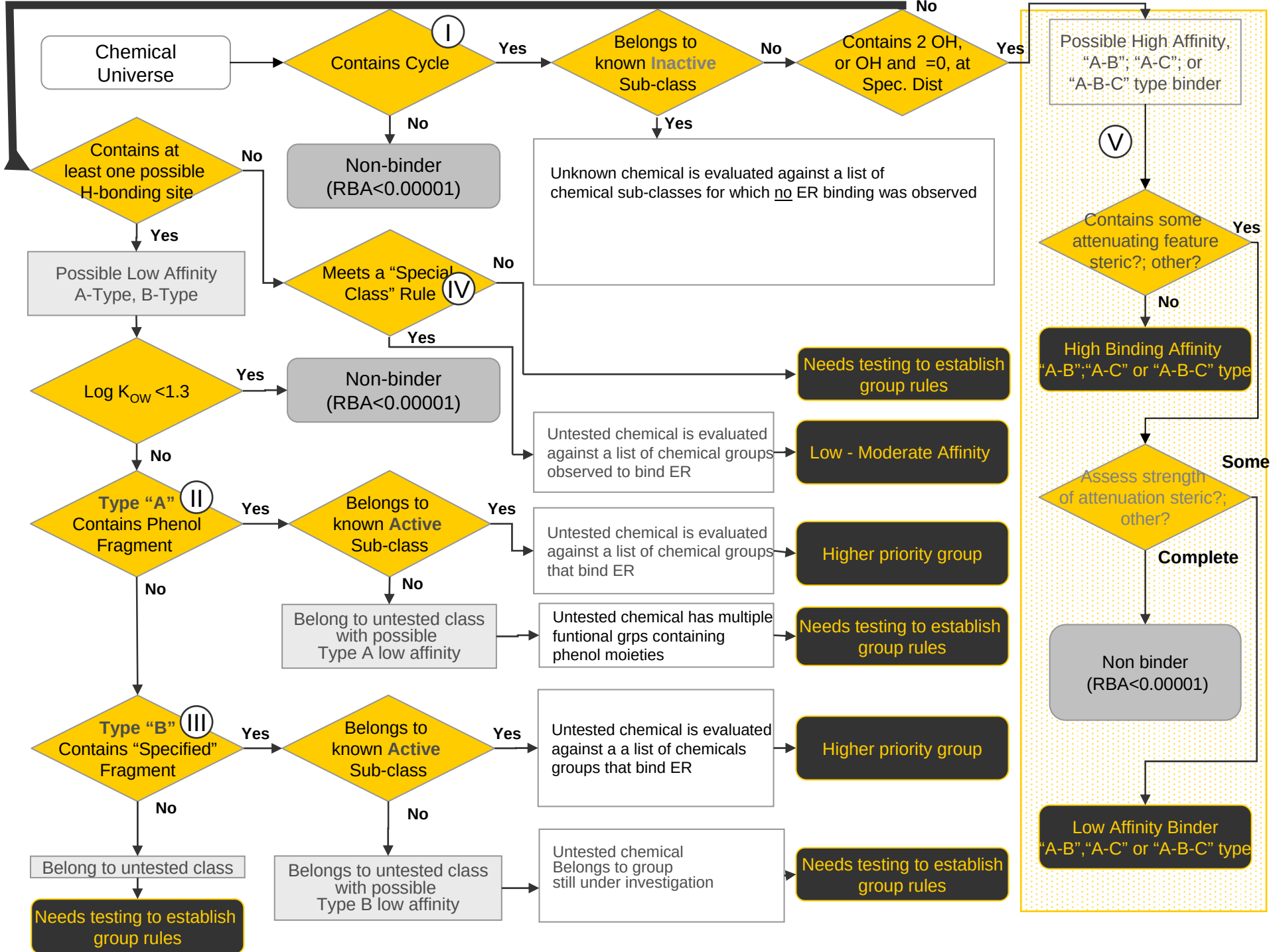
Food Use Inerts		Antimicrobials
393	*Total Chemicals*	211
378 (96%)	*Lower Probability*	196 (93%)
15 (4%)	*Higher Probability*	15 (7%)

ER Binders


Estradiol
Ethinyl Estradiol
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
Log(RBA)
0
1
2
3
4
5
6
7
8
Log(Kow)
Inerts
Antimicrobials

Adverse Outcome Pathway
ER-mediated Reproductive Impairment
Measurements made across levels of biological organization




Chemical Universe
I
Contains Cycle
Yes
Belongs to known Inactive Sub-class
No
Contains 2 OH, or OH and =0, at Spec. Dist
Yes
No
Possible High Affinity, "A-B"; "A-C"; or "A-B-C" type binder
No
No
Yes
Yes
Contains at least one possible H-bonding site
Non-binder (RBA<0.00001)
Unknown chemical is evaluated against a list of chemical sub-classes for which no ER binding was observed
V
Contains some attenuating feature steric?; other?
Yes
No
Possible Low Affinity A-Type, B-Type
Meets a "Special Class" Rule
IV
No
Yes
High Binding Affinity "A-B";"A-C" or "A-B-C" type
Needs testing to establish group rules
Log K_{OW} <1.3
Yes
Non-binder (RBA<0.00001)
Untested chemical is evaluated against a list of chemical groups observed to bind ER
Low - Moderate Affinity
No
Some
Assess strength of attenuation steric?; other?
Type "A" Contains Phenol Fragment
II
Yes
Belongs to known Active Sub-class
Yes
Untested chemical is evaluated against a list of chemical groups that bind ER
Higher priority group
Complete
No
No
Belong to untested class with possible Type A low affinity
Untested chemical has multiple funtional grps containing phenol moieties
Needs testing to establish group rules
Non binder (RBA<0.00001)
Type "B" Contains "Specified" Fragment
III
Yes
Belongs to known Active Sub-class
Yes
Untested chemical is evaluated against a a list of chemicals groups that bind ER
Higher priority group
No
No
Belong to untested class
Belongs to untested class with possible Type B low affinity
Untested chemical Belongs to group still under investigation
Needs testing to establish group rules
Low Affinity Binder "A-B","A-C" or "A-B-C" type
Needs testing to establish group rules

Summary

- Hypothesis-driven approach
  - Adverse Outcome pathway (*in vitro* linked to *in vivo*)
  - Strategic chemical selection and testing to cover types of chemicals found on the list that needed prioritizing
  - Mechanistic hypothesis (LogKow; low affinity binding types)

- Derived a QSAR-based Decision Support System that can be applied to next chemical list, and expanded where needed (chemical classes not yet tested)

- Developed priority setting tool to focus on the 4 to 7% of chemicals with plausible toxicological potential for an important adverse outcome.

Developing an Approach and Tools to move EPA toward the new paradigm