Supporting Information for Publication

Analysis of Eight Oil Spill Dispersants Using Rapid, In Vitro Tests for Endocrine and Other Biological Activity

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Appendix A – Oil Spill Dispersants and Reference Chemicals

Appendix A.1: Oil Spill Dispersants

The eight oil spill dispersants analyzed are listed in **Table S1**. Samples were shipped directly from the manufacturers to the EPA for aliquoting and shipping to the laboratories performing assays. All of these dispersants are on the National Contingency Plan product schedule at http://www.epa.gov/oem/content/ncp/product_schedule.htm. Toxicity values for aquatic species and efficacy values are also compiled by the EPA Office of Emergency Management at http://www.epa.gov/emergencies/content/ncp/tox_tables.htm.

Sample Name	Sample ID	EPA ID	Volume Received	Comments	Date Received	Manufacturer/ Source	Lot #	Storage
Corexit 9500	TX000362	G	1 L	hazy yellow	11-May-10	Nalco	10-May-10	D377A/J07022
JD 2000	TX006501	A	10 ml	clear yellow	27-May-10	Ethox Chemicals, LLC	20C366	D377A/J07022
DISPERSIT SPC 1000	TX006499	B	10 ml	clear amber	27-May-10	Polychem	NA	D377A/J07022
Sea Brat #4	TX006500	C	10 ml	hazy yellow	27-May-10	Alabaster Corp	NA	D377A/J07022
Nokomis 3-AA	TX006505	D	10 ml	clear light color	27-May-10	MAR-LEN Supply inc.	NA	D377A/J07022
Nokomis 3-F4	TX006504	E	10 ml	clear light color	27-May-10	MAR-LEN Supply inc.	NA	D377A/J07022
ZI-400	TX006518	H	25 ml	clear yellow	29-May-10	ZI Chemical	NA	D377A/J07022
SAF-RON GOLD	TX006258	I	500 ml	silver iridescent	4-June-10	Sustainable Environmental Technologies, Inc.	NA	D377A/J07022

Table S1 – Dispersant samples analyzed.

Corexit 9500: The major dispersant used during the 2010 oil spill incident in the Gulf of Mexico, Corexit 9500 is produced by Nalco Energy Services, L.P. (Sugar Land, Texas). The exact composition is proprietary, but some information is available from the EPA (http://www.epa.gov/bpspill/dispersants.html#bpdata), the material safety data sheet (MSDS; http://lmrk.org/corexit_9500_uscueg.539287.pdf) and other publications. The composition listed on the MSDS is: Distillates, petroleum, hydrotreated light (CAS No. 64742-47-8) 10.0 - 30.0%, Propylene Glycol (CAS No. 57-55-6) 1.0 - 5.0% and a proprietary organic sulfonic acid salt, 10.0 - 30.0%. A publication from Exxon also mentions that Corexit 9500 contains Tween 80 (eicosethoxy sorbitan monooleate) and Span 80, (ethoxylated sorbitan mono- and trioleates)[1] both of which are surfactants. U.S. Patent 6168702 has been reported to cover the class of sulfonic acid surfactants.

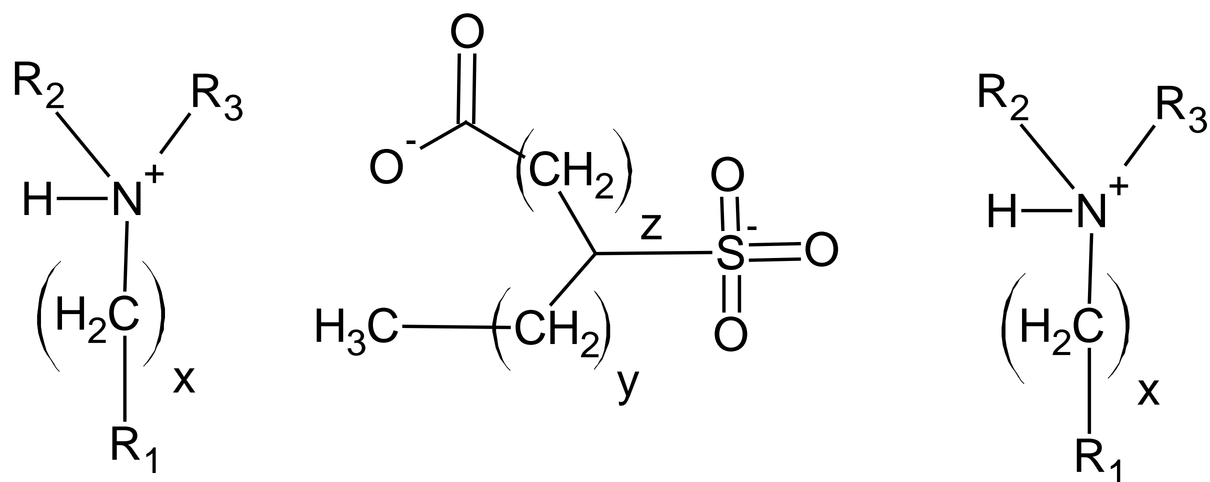

Figure S1 – reported structure for the class of surfactants used in Corexit 9500, as reported in U.S. Patent 6168702. wherein R_1 is H or an alkoxide of from 5 to about 20 carbon atoms; x is an integer of from about 8 to about 22 when R_1 is hydrogen and from about 2 to about 5 when R_1 is alkoxide; R_2 is independently selected from the group consisting of H, $(CH_2\ CH_2\ O)_m$ H, and $(CH_2\ CH(CH_3)O)_m$ H; R_3 is independently selected from the group consisting of H, $(CH_2\ CH_2\ O)_n$ H, and $(CH_2\ CH(CH_3)O)n$ H; M and n are integers from 1 to 50; and y and z are integers ranging from 2 to 10.

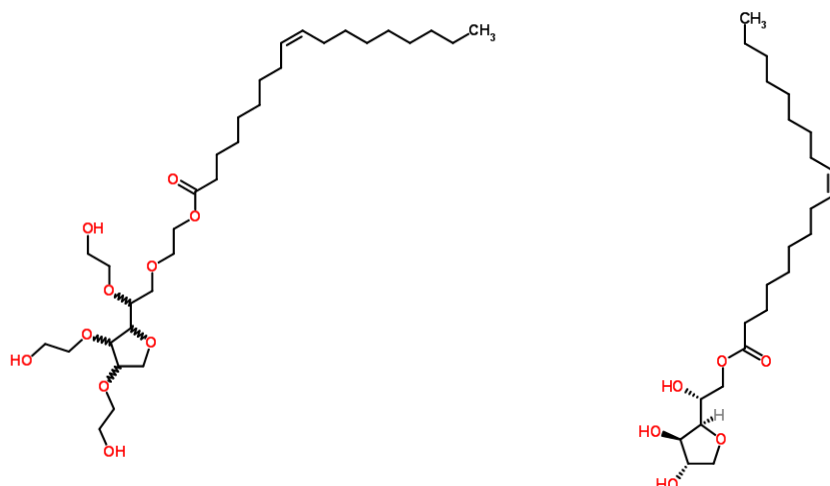

Figure S2: Chemical Structure of Tween 80 (Polysorbate 80, CAS No. 9005-65-6) and Span 80 (Sorbitan, mono-(9Z)-9-octadecenoate, CAS No. 1338-43-8)

Item	CAS Registry Number	Chemical Name (TSCA Inventory)
1	57-55-6	1,2-Propanediol
2	111-76-2	Ethanol, 2-butoxy-
3	577-11-7	Butanedioic acid, 2-sulfo-, 1,4-bis(2-ethylhexyl) ester, sodium salt (1:1)
4	1338-43-8	Sorbitan, mono-(9Z)-9-octadecenoate
5	9005-65-6	Sorbitan, mono-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs.
6	9005-70-3	Sorbitan, tri-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs
7	29911-28-2	2-Propanol, 1-(2-butoxy-1-methylethoxy)-
8	64742-47-8	Distillates (petroleum), hydrotreated light

Table S2: The following non-confidential component list for Corexit 9500 was provided by EPA at http://www.epa.gov/bpspill/dispersants.html#bpdata.

JD 2000: Manufactured by GlobeMark Resources. They provide an MSDS (http://globemarkresources.com/JD2000.php), but all information on the chemical including CAS No. and composition is listed as proprietary.

DISPERSIT SPC 1000: Manufactured by PolyChem. The composition is listed as “Aqueous product containing emulsifiers, dispersants, water dilutable coupling solvent”. There is a patent (U.S. Patent 6,261,463) that appears to pertain to this product, having the following introduction:

"An effective and non-toxic oil spill dispersant combines a predominately oil-soluble surfactant (e.g., polyethylene glycol mono-oleate) with a predominately water-soluble surfactant (e.g., cocoamide) and a co-solvent for coupling a mixture of the predominately oil-soluble surfactant and the oil spill, with the predominately water-soluble surfactant. Water is included in the combination to help advance the interaction between the predominately oil-soluble surfactant and the predominately water-soluble surfactant as well as the co-solvent. The water component also helps reduce the viscosity of the dispersant to allow it to be pumped under pressure." http://www.uspoly.com/disspec.html.

Item	CAS Registry Number	Chemical Name (TSCA Inventory)
1	61791-00-2	Fatty acids, tall-oil, ethoxylated (Ethox TO9A)
2	61791-26-2	Amines, tallow alkyl, ethoxylated (Ethox TAM-5)
3	68603-42-9	Amides, coco, N,N-bis(hydroxyethyl) (Ninol 40CO)
4	84133-50-6	Alcohols, C12-14-secondary, ethoxylated (Tergitol 19-S-5)
5	68608-26-4	Sulfonic acids, petroleum, sodium salts (Petrolnate HL, sod.) (Treated as a linear alkyl sulfonate sodium salt with 25 CH_2 units)
7	34590-94-8	Dipropylene glycol monomethyl ether
8	68411-30-3	Alkylbezenesulfonate (C10-C13 LAS)

Table S3: The following non-confidential components list for DISPERSIT SPC 1000 is based on analysis of U.S. Patent 6,261,463.

Sea Brat #4: Manufactured by Alabaster Corporation. No composition information is available beyond the MSDS (http://www.inspectapedia.com/hazmat/SEA_BRAT_4_MSDS.pdf). http://www.alabastercorp.com/seabrat.htm

Nokomis 3-AA: Manufactured by Nokomis. This is an aerial applied dispersant described as a water based colloidal surface-active agent oil spill dispersant. No further composition information is provided. No MSDS was found. http://www.nokomis3.com/nokomis-3AA/

Nokomis 3-F4: Manufactured by Nokomis. This is a surface applied dispersant described as a water based colloidal surface active agent oil spill dispersant. No further composition information is provided. No MSDS was found. http://www.nokomis3.com/nokomis-3F4/

ZI-400: Manufactured by ZI Chemicals. No information on composition was found, but an MSDS is available (http://www.interchem.co.tt/ZI400MSDS.pdf) indicating that this contains an alkali detergent (http://www.zichemicals.com/ZI400_OSD_Data.pdf).

Item	CAS Registry Number	Chemical Name (TSCA Inventory)
1	25155-30-0	Alkylbenzene sulfonic acid as dodecylbenzenesulfonic acid
2	9002-92-0	Alcohol Ethoxylate as Alcohols C9-11 Ethoxylated [Used Poly(oxy-1,2-ethanediyl), alpha-dodecyl-omega-hydroxy-]
3	1300-72-7	Sodium Xylene Sulfonate
4	68412-54-4	Nonionic Surfactant as Polyethylene Glycol Mono-P-Nonylphenyl Ether (Used poly(oxy-1,2-ethanediyl), alpha-(nonylphenyl)-omega-hydroxy-, branched)
5	Unavailable	Organic Sulfonates (unspecified)
6	Unavailable	Impurities (unspecified)

Table S4: Components of ZI-400 obtained from Australia's "Study of Health Outcomes in Aircraft Maintenance Personnel" available at http://www.defence.gov.au/health/research/shoamp/docs/Lit_Rev_Final_version_Sept2_2003.pdf.

SAF-RON GOLD: Manufactured by Saf-Ron Intl. an MSDS is available at http://www.saf-ron.com/PDFs/MSDSSAF-RONGOLD.pdf, but no information on composition or toxicity is provided.

Appendix A.2: Reference Chemicals

Most reference chemicals were selected from those recommended by ICCVAM for qualifying ER and AR binding and transactivation assays[2]. All of the chemical samples listed in **Table S5** were solubilized in DMSO and shipped to laboratories performing assays. Information on ER and AR activity derived from the ICCVAM report[2] is provided in **Table S9**.

Chemical Name	Sample ID	CASRN	Lot Number	Date Prep.	Purity	Supplier
17β-Estradiol	TX006457	50-28-2	010M0142	11-Jun-10	98	Sigma Aldrich
17β-Estradiol	TX006465	50-28-2	010M0142	11-Jun-10	98	Sigma Aldrich
17β-Trenbolone	TX006461	10161-33-8	020M1273	11-Jun-10	95	Sigma Aldrich
17β-Trenbolone	TX006467	10161-33-8	020M1273	11-Jun-10	95	Sigma Aldrich
17β-Trenbolone	TX006498	10161-33-8	71k1110	28-May-10	95	NHEERL
2,4,5-T	TX006450	93-76-5	SZE7303X	11-Jun-10	99	Sigma Aldrich
4-(tert-octyl)Phenol	TX006455	140-66-9	MKBB8667	11-Jun-10	97	Sigma Aldrich
4-Hydroxytamoxifen	TX006456	68392-35-8	020M4068	11-Jun-10	98	Sigma Aldrich
4-Nonylphenol (linear) (NP)	Toxcast_596	104-40-5	LB38315	14-Sep-06	98.5	Sigma Aldrich
4-Nonylphenol (branched) (NP)	TX006468	84852-15-3	07427BJ	11-Jun-10		Sigma Aldrich
5α-androstan-17β-ol-3-one	TX006460	521-18-6	1439199	11-Jun-10	97.5	Sigma Aldrich
5α-androstan-17β-ol-3-one	TX006466	521-18-6	1439199	11-Jun-10	97.5	Sigma Aldrich
Atrazine	Toxcast_13	1912-24-9	S03-2797	27-Jul-06	96.2	EPA OPP
Atrazine	TX006445	1912-24-9	SZE8175X	11-Jun-10	98.8	Sigma Aldrich
Bicalutamide	TX006451	90357-06-5	010M47452	11-Jun-10	98	Sigma Aldrich
Bisphenol A	TX006458	80-05-7	MKAA2480	11-Jun-10	99	Sigma Aldrich
Bisphenol A	TX006497	80-05-7	03105ES	28-May-10	99	NHEERL
Butyl benzyl phthalate	TX006444	85-68-7	SZE8156X	11-Jun-10	97.6	Sigma Aldrich
Butyl benzyl phthalate	TX006496	85-68-7	03405JH	28-May-10		NHEERL
Cyproterone acetate	TX006452	427-51-0	025K1270	11-Jun-10	98	Sigma Aldrich
Cyproterone acetate	TX006495	427-51-0	81K1186	28-May-10	98	NHEERL

DDE-p,p'	TX006454	72-55-9	11923EO	11-Jun-10	99	Sigma Aldrich
DDE-p,p'	TX006491	72-55-9	D397	28-May-10	99.9	NHEERL
Di-n-butyl phthalate	TX006462	84-74-2	SZE8149X	11-Jun-10	99.8	Sigma Aldrich
Di-n-butyl phthalate	TX006494	84-74-2	91997P5	28-May-10		NHEERL
Flutamide	TX006459	13311-84-7	08K1294	11-Jun-10		Sigma Aldrich
Flutamide	TX006493	13311-84-7	87H1511	28-May-10		NHEERL
Genistein	TX006453	446-72-0	098K0735	11-Jun-10	98	Sigma Aldrich
Igepal CO-210 (NPE)	TX006470	68412-54-4	MKBC8907	11-Jun-10		Sigma Aldrich
Linuron	TX006446	330-55-2	5320X	11-Jun-10	99.7	Sigma Aldrich
Linuron	TX006492	330-55-2	265-44A	28-May-10		NHEERL
Methoxychlor	TX006449	72-43-5	SZE8289X	11-Jun-10	98.7	Sigma Aldrich
Procymidone	TX006448	32809-16-8	SZE8158X	11-Jun-10	99.9	Sigma Aldrich
Procymidone	TX006490	32809-16-8	231-100A	28-May-10	99	NHEERL
Tergitol NP-9 (NPE)	TX006469	127087-87-0	07409BH	11-Jun-10		Sigma Aldrich
Vinclozolin	TX006447	50471-44-8	SZE7292X	11-Jun-10	99.5	Sigma Aldrich
Vinclozolin	TX006489	50471-44-8	2296X	28-May-10	99.6	NHEERL

Table S5: Reference chemicals used for NCCT studies of dispersants.

Appendix B – Assay Protocols

Appendix B.1: Cis and Trans Transcription Factor Activity Reporter Gene Assays

Data on a large collection of transcription factor assays, including two ER assays, were run on the samples. This collection of a multiplexed reporter gene assays and data on 309 environmental chemicals are described by Martin et al.[3]. Attagene Inc. (RTP, NC), under contract to the U.S. EPA (Contract Number EP-W-07-049), provided multiplexed reporter transcription unit (RTU) assays (Factorial, patents pending) consisting of 48 human transcription factor DNA binding sites transfected into the HepG2 human liver hepatoma cell line as previously described[4]. In addition to the cis-acting reporter genes (CIS), a modification of the approach was used to generate a trans-system (TRANS) with a mammalian one-hybrid assay consisting of an additional 25 RTU library reporting the activity of nuclear receptor (NR) superfamily members[5]. The human ligand-binding domain of each nuclear receptor was expressed as a chimera with the yeast GAL4 DNA-binding domain that activated in trans a 5XUAS-TATA promoter, which regulated the transcription of a reporter sequence unique to each NR RTU. To ensure the specificity of detection, each individual trans-RTU system including both receptor and reporter gene was separately transfected into suspended cells followed by pooling and plating of the transfected cells prior to screening. A major difference between the CIS and TRANS system is that in CIS activities of endogenous transcription factors are measured, whereas the TRANS assay evaluates changes in activities of exogenous, chimeric NR-Gal4 proteins. Since the HepG2 cell line does not express some nuclear receptors, the CIS assay cannot be used to evaluate these targets, including the androgen receptor. A cytotoxicity assessment was performed at the higher concentrations, qualitatively, to remove confounding data from the downstream analysis process. Further assessment was performed at all concentrations by testing the samples for cytotoxicity against HepG2 cells in the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay following 24 hour exposure. An LC50 was determined to further filter out data confounded by cytotoxicity. The following tables list the complete set of CIS and TRANS assays run as part of this study.

RTU Name	ASSAY NAME	Induced by Transcription Factors/ Inducers	Biological Pathways
AhRE	ATG_Ahr_CIS	AhR / Dioxin, FIC	Xenobiotic response, hypoxia
AP-1	ATG_AP_1_CIS	AP-1-like components: c-fos, c-jun / PMA	JNK pathway, stress responses
AP-2	ATG_AP_2_CIS	AP-2 Family	Embryonic morphogenesis
BRE	ATG_BRE_CIS	SMAD Family / BMPs	Osteoblast differentiation
C/EBP	ATG_C_EBP_CIS	C/EBP Family	Adipogenesis
CMV	ATG_CMV_CIS	Cytomegalovirus (CMV) promoter	Negative Control (Viral Promoter)
CRE	ATG_CRE_CIS	CREB Family / Forskolin, cAMP	cAMP, cGMP, NO receptor, GPCR pathways
LXRE (DR4)	ATG_DR4_LXR_CIS	LXRα, β/oxysterols	Nuclear hormone receptor pathway
RARE(DR5)	ATG_DR5_CIS	RARα,β,γ/ Retinoic acid	Nuclear hormone receptor pathway
E-box	ATG_E_Box_CIS	c-Myc, USF1	Cell cycle, proliferation
E2F	ATG_E2F_CIS	E2F subfamily	Cell cycle, Proliferation
EGR	ATG_EGR_CIS	Egr subfamily/ Growth factors	Receptor tyrosine kinase pathway
ERE	ATG_ERE_CIS	ER/estragens	Nuclear hormone receptor pathway
Ets	ATG_Ets_CIS	Ets Family/ PMA	MAP kinase mediated signaling
FoxA	ATG_FoxA2_CIS	Fork Head family: FoxA	Hepatic specification
FoxO	ATG_FoxO_CIS	Fork Head Family: FoxO	Akt/Forkhead signaling
GATA	ATG_GATA_CIS	GATA Family	Differentiation
GLI	ATG_GLI_CIS	Gli subfamily	Hedgehog pathway
GRE	ATG_GRE_CIS	GR / Dexamethazone	Differentiation, inflammation
HIF1α	ATG_HIF1a_CIS	HIF1α/ hypoxia	Hypoxia, angiogenesis
HNF6	ATG_HNF6_CIS	Tissue specific regulator: HNF6	Endocrine cell differentiation
HSE	ATG_HSE_CIS	HSF Family / heat shock, geldanamycin	Stress response, heat shock
FXRE (IR1)	ATG_IR1_CIS	preferential binding site for FXR	Nuclear hormone receptor pathway

ISRE	ATG_ISRE_CIS	Interferone regulating factors: IRF1, IFR3 / Interferones	Immune responses, host defense
MRE	ATG_MRE_CIS	MTF-1/ heavy metals	Heavy metals response
Myb	ATG_Myb_CIS	Myb Family	Hematopoietic cell differentiation
Myc	ATG_Myc_CIS	specific to c-Myc	Proliferation, apoptosis
NF-kB	ATG_NF_kB_CIS	Rel Family/ TNFα, IL-1β, LPS	Immune responses, IL-1/Toll receptor pathway
NFI	ATG_NFI_CIS	NF-1 family	Drug metabolism
NRF1	ATG_NRF1_CIS	NFE2 subfamily: NRF1	Mitochondria genesis, antioxidative responses
ARE	ATG_NRF2_ARE_CIS	NF-E2 subfamily:NRF2	Antioxidative responses
Oct	ATG_Oct_MLP_CIS	POU domain Family: OCTs	Development
p53	ATG_p53_CIS	p53 family/ DNA damage	Genotoxics stress responses
Pax	ATG_Pax6_CIS	Pax Family	Development of CNS, beta cell differentiation
PBREM	ATG_PBREM_CIS	CAR/ Phenobarbetal	Nuclear hormone receptor pathway
PPRE	ATG_PPRE_CIS	PPARα, δ, γ / Rosiglitazone	Nuclear hormone receptor pathway
PXRE	ATG_PXRE_CIS	PXR / Rifampicin	Nuclear receptor pathway
RORE	ATG_RORE_CIS	partially specific for RORs α,β,γ	Nuclear receptor pathway
SOX	ATG_Sox_CIS	SOX Family	Chondrogenesis
Sp1	ATG_Sp1_CIS	Ubiquitous transcription factor Sp1	Differentiation
SREBP	ATG_SREBP_CIS	SREBP subfamily	Lipid homeostasis
STAT	ATG_STAT3_CIS	STAT Family / IL-6	JAK pathway
TA	ATG_TA_CIS	MHC2 TA promoter	Negative Control (Minimal Promoter)
TAL	ATG_TAL_CIS	TAL promoter	Negative Control (Minimal Promoter)
TCF/β-cat	ATG_TCF_b_cat_CIS	TCF-1 Family/ Wnt	Cell adhesion, Wingless-Int pathway
TGFβ	ATG_TGFb_CIS	SMAD Family / TGFb	Cell growth and differentiation TGFb pathway
VDRE	ATG_VDRE_CIS	VDR/ vitamin D	Nuclear receptor pathway
Xbp1	ATG_Xbp1_CIS	X-Box protein 1	Unfolded protein response, ER stress

Table S6 – Listing of CIS assays run by Atttagene, including a short description of the molecular target's biological pathway.

RTU Name	ASSAY NAME	Name of Receptor (Gene Symbol)
AR	ATG_AR_TRANS	Androgen Receptor (NR3C4)
CAR	ATG_CAR_TRANS	Constitutive androstane receptor (NR1I3)
ERa	ATG_ERa_TRANS	Estrogen receptor alpha (NR3A1)
ERRa	ATG_ERRa_TRANS	Estrogen-related receptor alpha (NR3B1)
ERRg	ATG_ERRg_TRANS	Estrogen-related receptor gamma (NR3B3)
FXR	ATG_FXR_TRANS	Farnesoid X-activated receptor (NR1H4)
GR	ATG_GR_TRANS	Glucocorticoid receptor (NR3C1)
Hpa5	ATG_Hpa5_TRANS	Human platelet antigen 5 (negative control)
LXRa	ATG_LXRa_TRANS	Liver X receptor alpha (NR1H3)
LXRb	ATG_LXRb_TRANS	Liver X receptor beta (NR1H2)
NHF4a	ATG_HNF4a_TRANS	Hepatocyte nuclear factor 4-alfa (NR2A1)
NURR1	ATG_NURR1_TRANS	Nur-related protein 1 (NR4A2)
PPARa	ATG_PPARa_TRANS	Peroxisome proliferator activated receptor alpha (NR1C1)
PPARd	ATG_PPARd_TRANS	Peroxisome proliferator activated receptor delta (NR1C2)
PPARg	ATG_PPARg_TRANS	Peroxisome proliferator activated receptor gamma (NR1C3)
PXR	ATG_PXR_TRANS	Pregnane X receptor (NR1I2)
RARa	ATG_RARa_TRANS	Retinoic acid receptor alpha (NR1B1)
RARb	ATG_RARb_TRANS	Retinoic acid receptor beta (NR1B2)
RARg	ATG_RARg_TRANS	Retinoic acid receptor gamma (NR1B3)
RORb	ATG_RORb_TRANS	RAR-related orphan receptor beta (NR1F2)
RORg	ATG_RORg_TRANS	RAR-related orphan receptor gamma (NR1F3)
RXRa	ATG_RXRa_TRANS	Retinoid X receptor alpha (NR2B1)
RXRb	ATG_RXRb_TRANS	Retinoid X receptor (NR2B2)
THRa	ATG_THRa1_TRANS	Thyroid hormone receptor alpha (NR1A1)
VDR	ATG_VDR_TRANS	Vitamin D3 receptor (NR1I1)

Table S7 – Listing of TRANS assays run by Attagene

Appendix B.2: AR beta-lactamase Assay (agonist and antagonist modes)

Overview: The androgen receptor (AR), located at chromosome Xq11-12, is an important member of nuclear hormone receptors. AR signaling plays a critical role in AR-dependent prostate cancer and other androgen related diseases. Considerable attention has been given in the past decades to the possibility of some of environmental chemicals which may contribute the endocrine disruption. Therefore, it is important to understand the effect of environmental chemicals in AR signaling pathway.

NCGC Assay Protocol Summary: Using a beta-lactamase reporter-gene under control of the AR response element, a cell-based assay [GeneBLAzer® AR-UAS-bla-GripTite™ assay developed by Invitrogen] was used to measure AR ligands signaling. AR-UAS-bla-GripTite™ HEK 293 cells (AR *bla* cells) were used with assay medium containing 10% dialyzed FBS, 0.1 mM NEAA and 1 mM sodium pyruvate. The assay was performed in clear bottom black Greiner 1536-well plates. R1881, a synthetic androgen agonist, was used as a positive control in the screen. Library compounds were measured for their ability to either stimulate or inhibit the reporter gene activity. Compounds were screened in a titration series in 1536-well format. The fluorescence intensity (405 nm excitation, 460/530 nm emission) was measured using an EnVision plate reader. Data was normalized relative to R1881 control (40 nM, 100%, for agonist mode and 10 nM, 0%, for antagonist mode), and DMSO only wells (basal, 0% for agonist mode and -100% for antagonist mode). Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations of half-maximal stimulation (EC_{50}), half-maximal inhibition (IC_{50}) and maximal response (efficacy) values.

qHTS protocol for AR beta-lactamase assay in agonist mode

[Step] [Parameter] [Value] [Description]

1. Reagent; 6 µL; 2000 cells/well
2. Incubation; 37ºC for 5 hrs
3. Reference compounds; 23 nL; 9.1 pM to 76.7 µM; dispersants; 23 nL; 0.00012 ppm to 1008 ppm
4. Controls (R1881 and DMSO); 23 nL,

   a. Final concentrations of R1881 of 20 nM in row AC (wells 1-24), 40 nM in row AD (wells 1-24), 80 nM in row AC (wells 25-48) and 160 nM in row AD (wells 25-48)
   b. DMSO in row AE and AF
5. Incubation; 37ºC for 16 hrs
6. Detection reagent; 1 μL of CCF4-dye
7. Incubation; RT for 1.5 hrs
8. Detection; Fluorescence; Envision plate reader (PerkinElmer)

qHTS protocol for AR beta-lactamase assay in antagonist mode

[Step] [Parameter] [Value] [Description]

1. Reagent; 5 μL; 2000 cells/well
2. Incubation; 37ºC for 5 hrs
3. Compounds; 23 nL; 9.1 pM to 76.7 μM; dispersants; 23 nL; 0.00012 ppm to 1008 ppm
4. DMSO Control; 23 nL,
5. Agonist (Stimulator); 1 μL; R1881 at 10nM (final) concentration
6. Incubation; 37ºC for 16 hrs
7. Detection reagent; 1 μL of CCF4-dye
8. Incubation; RT for 1.5 hrs
9. Detection; Fluorescence; Envision plate reader (PerkinElmer)

Cytotoxicity Analysis: Cell viability after compound treatment was measured in these AR *bla* cells using a luciferase-coupled ATP quantitation assay (CellTiter-Glo viability assay, Promega). The change of intracellular ATP content indicates the number of metabolically competent cells after compound treatment. The cells were dispensed at 2,000 cells/5 μL/well for AR *bla* cells in 1,536-well white/solid bottom assay plates using an FRD. The cells were incubated for 5 hrs at 37°C, followed by the addition of compounds using the pin tool. The final concentration range for reference compounds was 11 pM to 92 μM, and 0.000144 ppm to 1209.8 ppm for dispersants. The assay plates were incubated for 16 hrs at 37°C, followed by the addition of 5 μL/well of CellTiter-Glo reagent. After 30 min incubation at room temperature, the

luminescence intensity of the plates was measured using a ViewLux plate reader (PerkinElmer). Data was normalized relative to DMSO only wells (0%), and tetra-n-octylammonium bromide (92 µM, -100%). Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations of half-maximal cell death (IC_{50}) and the associated maximal percent cell-death. Using these curve fits, a sensitive measure of cytotoxicity, twenty-percent cell-death or an IC_{20}, was calculated. These values were then used to filter out ER and AR activity at or above the cytotoxicity threshold.

Appendix B.3: ER beta-lactamase Assay (agonist and antagonist modes)

Overview: The estrogen receptor alpha (ERα) is an important member of nuclear hormone receptors. ERα plays important role in breast cancer as well as in other women's health issues. The majority of breast cancers is estrogen receptor (ER) positive and depends on estrogen for growth. There is also growing concern for some of environmental chemicals which may contribute the increasing the incidence of breast cancer. Therefore, it is important to understand the effect of environmental chemicals in ERα signaling pathway.

NCGC Assay Protocol Summary: Using a beta-lactamase reporter-gene under control of the ER response element, a cell-based assay [ERα-UAS-bla GripTite^{TM} cell-Based Assay from Invitrogen] was used to measure ERα signaling pathway both in agonist and antagonist modes. ERα-UAS-bla-GripTite™ HEK 293 cells (ERα *bla* cells) were used with assay medium containing 2% charcoal/dextran treated FBS, 0.1 mM NEAA and 1 mM sodium pyruvate. Cells were cultured in this assay medium overnight in the flasks before the assay. The assay was performed in clear bottom black Greiner 1536-well plates. 17β-Estradiol was used as a positive control in the screen. Library compounds were measured for their ability to either stimulate or inhibit the reporter gene activity. Compounds were screened in a titration series in 1536-well format. The fluorescence intensity (405 nm excitation, 460/530 nm emission) was measured using an EnVision plate reader. Data was normalized relative to 17β-Estradiol control (20 nM, 100%, for agonist mode and 0.5nM, 0%, for antagonist mode), and DMSO only wells (basal, 0% for agonist mode and -100% for antagonist mode). Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations of half-maximal stimulation (EC_{50}), half-maximal inhibition (IC_{50}) and maximal response (efficacy) values.

qHTS protocol for ER beta-lactamase assay in agonist mode

[Step] [Parameter] [Value] [Description]

1. Reagent; 6 µL; 5000 cells/well
2. Incubation; 37°C for 5 hrs
3. Compounds; 23 nL; 9.1 pM to 76.7 µM; dispersants; 23 nL; 0.00012 ppm to 1008 ppm

4. Controls (17β-Estradiol and DMSO); 23 nL
    a. Final concentrations of 17β-Estradiol of 40 in row AD and 20 nM in row AE
    b. DMSO in row AF
5. Incubation; 37ºC for 18 hrs
6. Detection reagent; 1 μL of CCF4-dye
7. Incubation; RT for 1 to 1.5 hrs
8. Detection; Fluorescence; Envision plate reader (PerkinElmer)

qHTS protocol for ER beta-lactamase assay in antagonist mode

[Step] [Parameter] [Value] [Description]

1. Reagent; 5 μL; 5000 cells/well
2. Incubation; 37ºC for 5 hrs
3. Compounds; 23 nL; 9.1 pM to 76.7 μM; dispersants; 23 nL; 0.00012 ppm to 1008 ppm
4. DMSO Control; 23 nL
5. Agonist (Stimulator); 1uL; 17β-Estradiol at 0.5 nM (final) concentration
6. Incubation; 37ºC for 18 hrs
7. Detection reagent; 1 μL of CCF4-dye
8. Incubation; RT for 1-1.5 hrs
9. Detection; Fluorescence; Envision plate reader (PerkinElmer)

Cytotoxicity Analysis: Cell viability after compound treatment was measured in these ER *bla* cells using a luciferase-coupled ATP quantitation assay (CellTiter-Glo viability assay, Promega). The change of intracellular ATP content indicates the number of metabolically competent cells after compound treatment. The cells were dispensed at 5,000 cells/5 μL/well for ERα *bla* cells in 1,536-well white/solid bottom assay plates using an FRD. The cells were incubated a 5 h at 37°C, followed by the addition of compounds using the pin tool. The final concentration range for reference compounds was 11 pM to 92 μM, and 0.000144 ppm to 1209.8 ppm for dispersants. The assay plates were incubated for 18 hrs at 37°C, followed by the addition of 5 μL/well of CellTiter-Glo reagent. After 30 min incubation at room temperature, the luminescence intensity of the plates was measured using a ViewLux plate reader (PerkinElmer). Data was normalized relative to DMSO only wells (0%), and tetra-n-octylammonium bromide

(92 uM, -100%). Concentration-response titration points for each compound were fitted to the Hill equation yielding concentrations of half-maximal cell death (IC_{50}) and the associated maximal percent cell-death. Using these curve fits, a sensitive measure of cytotoxicity, twenty-percent cell-death or an IC_{20}, was calculated. These values were then used to filter out ER and AR activity at or above the cytotoxicity threshold.

Appendix C – Curve Fitting Method

We used the concentration-response data from all assays (see Appendix B) to derive characteristic activity concentrations for each test compound, after first removing outliers and points beyond estimated thresholds of cytotoxicity. Viability assays were fit using the same methodology as stated below without cytotoxicity or outlier removal and required at least 20% cell death to achieve a characteristic (half-maximal) activity concentrations (AC_{50}). These curve fits were further analyzed to extract a twenty-percent lethal concentration (LC_{20}) for use as a cytotoxicity filter for associated assays.

First, in order to identify statistical outlier thresholds for each assay platform, all data were plotted to inspect data integrity and considered in the context of previous experience with the performance and dynamic range of these assays across a diverse set of chemicals[6]. For each concentration, d, outliers were defined as replicate response points, $Y_{d_{rep}}$, where:

$$\left|Y_{d_{rep}}\right| - \left(\left|\overline{Y}_d\right| \big/ MAD(Y_d) \right) \geq Threshold_{assay} \quad (1)$$

Following outlier removal, the data for each assay were filtered according to the corresponding cytotoxicity measures accompanying each assay. The cytotoxicity threshold for each assay was defined as the LC_{20}. The baseline was defined as the lower ("bottom") asymptote of the fitted cytotoxicity response.

Next, AC_{50} were calculated from the remaining concentration-response data points. Standard sigmoidal curves were fit using a 4-parameter Hill model. This model defines a response in terms of four parameters; T, B, AC_{50}, and Hillslope (W), as in the following equation:

$$f(X) = T - \frac{T - B}{1 + \left(X \big/ AC_{50} \right)^{W}} \quad (2)$$

The T and B are the upper (“top”) and lower (“bottom”) asymptotes of each assay response, respectively. The AC_{50} represents the inflection point in each response, thus providing a way to compare each compound’s potency across assays. The *W* parameter dictates the curve slope (response relative to concentration). The parameters were fit using the *nls()* function in R with the “port” algorithm (R Development Core Team, Vienna, 2010).

Along with careful inspection of all fit results, AC_{50} “hit” acceptance criteria were applied as a combination of efficacy ($E_{max} = \max(\bar{Y})$), response at the top asymptote of the sigmoidal fit (T), and goodness-of-fit (r^2). From previous experience with each assay platform’s performance across a broad set of chemicals[6], efficacy, model response, and goodness-of-fit thresholds were applied in an assay-specific manner. For the multiplexed reporter gene assays, hits were defined as $E_{max} > 2$ (fold-change), $T > 2$ (fold-change), $r2 > 0.5$; for the qHTS assays, hits were defined as $E_{max} > 20$ (% change), $T > 20$ (% change), $r2 > 0.5$; for the competitive binding assays, hits were defined as $E_{max} > 20$ (% inhibition), $T > 20$ (% inhibition), $r2 > 0.5$. As a quantification of the statistical significance of each response pattern, we present p-values from a standard two-sample t-test (assuming unequal variances). For the t-test, the two samples were defined from the data as the group of points representing the estimated top (T) versus bottom (B) asymptotes of the fitted model.

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Appendix D – Viability Data

ID	NAME	TARGET	AC50	CI (Lower)	CI (Upper)
B	Dispersit SPC 1000	ORD_Menidia_96hr	2.9	2.5	3.2
B	Dispersit SPC 1000	ORD_Mysid_48hr	12	10	14
E	Nokomis 3-F4	ORD_Menidia_96hr	19	16	21
D	Nokomis 3-AA	ORD_Menidia_96hr	20	18	22
H	ZI -400	ORD_Menidia_96hr	21	18	23
B	Dispersit SPC 1000	ATG_Viability	28	21	39
D	Nokomis 3-AA	ORD_Mysid_48hr	30	27	34
E	Nokomis 3-F4	ORD_Mysid_48hr	42	38	47
G	Corexit 9500	ORD_Mysid_48hr	42	38	47
I	SAF-RON GOLD	ORD_Menidia_96hr	44	41	47
C	Sea Brat 4	ORD_Menidia_96hr	55	49	62
H	ZI -400	ORD_Mysid_48hr	55	50	61
C	Sea Brat 4	ORD_Mysid_48hr	65	57	74
I	SAF-RON GOLD	ORD_Mysid_48hr	118	104	133
G	Corexit 9500	ATG_Viability	120	104	128
G	Corexit 9500	ORD_Menidia_96hr	130	122	138
B	Dispersit SPC 1000	NCGC_AR_Viability	170	160	172
E	Nokomis 3-F4	ATG_Viability	180	141	232
D	Nokomis 3-AA	ATG_Viability	200	166	228
D	Nokomis 3-AA	NCGC_AR_Viability	200	183	217
D	Nokomis 3-AA	NCGC_ERa_Viability	220	192	243
B	Dispersit SPC 1000	NCGC_ERa_Viability	260	236	283
G	Corexit 9500	NCGC_ERa_Viability	310	299	320
H	ZI-400	NCGC_ERa_Viability	340	300	381
E	Nokomis 3-F4	NCGC_AR_Viability	370	340	394
C	Sea Brat 4	ATG_Viability	410	361	ND
G	Corexit 9500	NCGC_AR_Viability	410	386	433
H	ZI-400	ATG_Viability	420	354	509
E	Nokomis 3-F4	NCGC_ERa_Viability	430	391	476
C	Sea Brat 4	NCGC_AR_Viability	560	515	603
C	Sea Brat 4	NCGC_ERa_Viability	580	516	638
H	ZI-400	NCGC_AR_Viability	670	620	719
A	JD 2000	ORD_Mysid_48hr	788	627	946
I	SAF-RON GOLD	NCGC_AR_Viability	1400	ND	ND
A	JD 2000	NCGC_AR_Viability	3200	3140	4730
A	JD 2000	ATG_Viability	>1000	ND	ND

I	SAF-RON GOLD	ATG_Viability	>1000	ND	ND
A	JD 2000	NCGC_ERa_Viability	>1000	ND	ND
I	SAF-RON GOLD	NCGC_ERa_Viability	>1000	ND	ND
A	JD 2000	ORD_Menidia_96hr	>1000	ND	ND

Table S8: Viability data for the dispersants for all in vitro assays and the EPA ORD small fish and brine shrimp data[7]. All values are in ppm. ND= not determined, i.e. AC50 above highest concentration tested. The entries in the table are ordered from most toxic (top) to least toxic (bottom).

Appendix E – Reference Chemical Data for ER Assays

This study uses a battery of ER assays, and EPA has published guidelines for validating such assays for use in the Endocrine Disruptor Screening Program (EDSP). Although the assays used here are not validated, we can compare their performance characteristics with that required for validated assays, in particular by a comparison with expected results on reference chemicals. For this report, we have only carried out this comparison for the ER assays used because no AR activity was seen in any of the dispersants.

This study used 19 chemicals that are reference chemicals for ER binding or transcriptional activation tests according to ICCVAM[2]. These are listed in **Table S9**, along with the expected results for transcriptional activation in the agonist mode, which is the activity seen for some of the dispersants. For each of the chemicals, we noted the expected activity from ICCVAM (see table legend for meaning of symbols) and where available, from the EPA OPPTS ER transactivation assay guideline[8]. AC_{50} and EMax values for the Attagene CIS and TRANS ER assays and the NCGC ER agonist assay are also given.

If the expected activity was seen, the cell was colored green and if the expected was not seen, the cell was colored red. In cases where the expected value was equivocal, or ICCVAM and OPPTS disagreed, no color was used. For the Attagene ERα TRANS assay, we agree with the ICCVAM call for positive calls in 9 cases, and for negative calls in six. The assay disagrees in one positive and one negative call. The disagreeing call for Trenbolone is confusing because this chemical is positive in both including a native estrogen receptor and the estrogen receptor ligand-binding domain, as well as the cell-free competitive-binding assay (data not shown). The ERE CIS assay has one more false positive call, for Vinclozolin. So, while not getting 100% agreement with the ICCVAM calls, these assays have a high rate of agreement for both negative and positive cases. One other point to note is that the Attagene ERα TRANS assay produces about twice the response (EMax) as the corresponding CIS assays, while the AC_{50} values are very similar.

Substance	CASRN	ICCVAM ER Agonist (a)	OPPTS ER Agonist	Attagene ERa TRANS		Attagene ERE CIS		NCGC ER Agonist	
				AC_{50}	EMax	AC_{50}	EMax	AC_{50}	EMax
17β-Trenbolone	10161-33-8	-		0.35	13	0.38	7.2	1.7	71
17β-Estradiol	50-28-2	+++	Pos	0.0025	20	0.00053	9.8	0.00029	110
Atrazine	1912-24-9	-	Neg	>100	NA	>100	NA	>100	NA
Bisphenol A	80-05-7	+		0.37	9.9	0.59	4.6	1.4	32
Butyl benzyl phthalate	85-68-7	++		40	6	47	6.1	>100	NA
Di-n-butyl phthalate	84-74-2	+	Neg	37	2.1	>100	NA	>100	NA
Flutamide	13311-84-7	-		>100	NA	>100	NA	>100	NA
Linuron	330-55-2	-		>100	NA	>100	NA	>100	NA
4-Nonylphenol (linear) (NP)	104-40-5	++		11	8.3	4.3	2.7	>100	NA
DDE-p,p'	72-55-9	+		29	9	48	4.1	>100	NA
Methoxychlor	72-43-5	+		2.3	6.4	17	6.5	4.2	30
Procymidone	32809-16-8	-		>100	NA	>100	NA	>100	NA
Vinclozolin	50471-44-8	-		>100	NA	19	4.8	>100	NA
2,4,5-T	93-76-5	+		>100	NA	>100	NA	>100	NA
Cyproterone acetate	427-51-0	-		>100	NA	>100	NA	4.9	31
Genistein	446-72-0	+	Pos	0.092	17	0.12	9.7	4.7	73
4-(tert-octyl)Phenol	140-66-9	++		2.5	9.8	3	5.8	1	88
4-Hydroxytamoxifen	68392-35-8	+-		>100	NA	>100	NA	58	68
5α-androstan-17β-ol-3-one	521-18-6	++		2.2	11	3.3	9.5	0.71	93
4-Nonylphenol (branched) (NP)	84852-15-3			0.68	12	0.61	5.4	0.42	59
Igepal CO-210 (NPE)	68412-54-4			2.5	8.5	14	6.5	0.49	87
Tergitol NP-9 (NPE)	127087-87-0			5.7	4.8	5.6	2.1	1.2	63

Table S9: Comparison of activity in the ER assays used in this study against the expectations for the ICCVAM and OPPTS ER reference chemicals analyses in the study.

(a) For the ICCVAM calls, +++ Indicates that the substance was strongly active (EC_{50} value was <0.001 µM); ++ indicates that the substance was moderately active (EC_{50} value was between 0.001 and 0.1 µM); + indicates that the substance was weakly active (EC_{50} value was >0.1 µM), or a positive response was reported without an EC_{50} value. The EC_{50} is the effective concentration that causes half-maximal activation of the receptor. +- indicates equivocal results in the literature[2].

Appendix F – P-value Matrix for Cytotoxicity Comparison

	JD 2000	Dispersit SPC 1000	Sea Brat 4	Nokomis 3-AA	Nokomis 3-F4	Corexit 9500	ZI-400	SAF-RON GOLD
JD 2000		5.3E-5	3.6E-5	5.3E-9	0.00011	0.00016	0.00039	1
Dispersit SPC 1000	1.9E-6		0.364	1	1	1	1	0.36
Sea Brat 4	1.3E-6	0.013		0.129	1	1	1	0.62
Nokomis 3-AA	1.9E-10	0.47	0.0046		1	1	1	0.36
Nokomis 3-F4	3.8E-6	0.16	0.11	0.19		1	1	0.45
Corexit 9500	5.7E-6	0.31	0.078	0.44	0.7		1	0.42
ZI-400	1.4E-5	0.054	0.74	0.054	0.3	0.21		0.59
SAF-RON GOLD	0.37	0.013	0.022	0.013	0.016	0.015	0.021	

Raw P-Value

Bonferroni Corrected P-Value

Table S10: Statistical comparison of LC50 cytotoxicity values from cell-based assays across the eight dispersants. All dispersants combinations with a p-value less <0.05 are shaded green. All values below the diagonal are raw p-values derived from the ANOVA, while all values above the diagonal were adjusted for multiple testing.

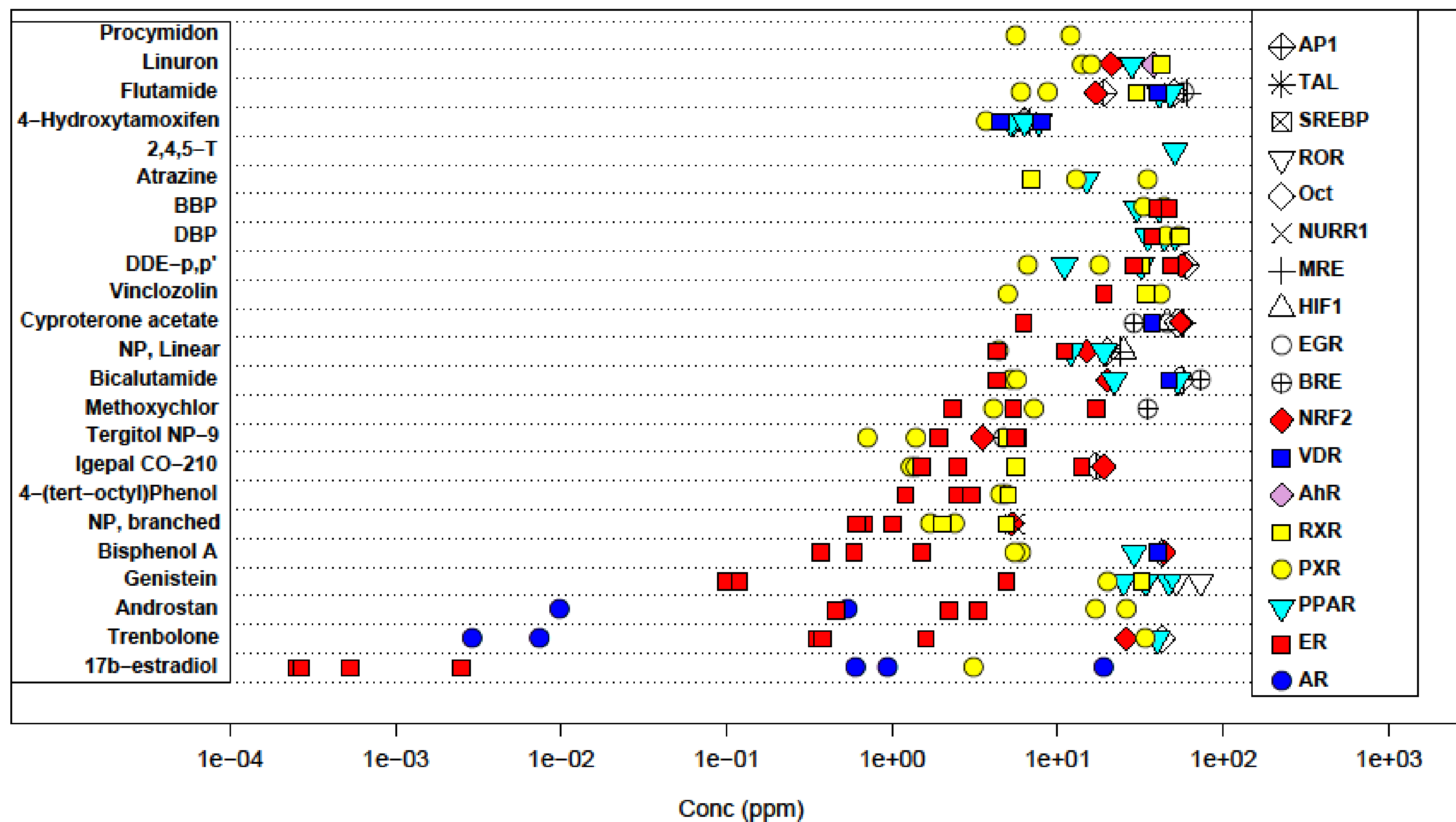

Figure S3: Summary plot of results for all Attagene and NCGC assays and the reference chemicals. Each horizontal band displays EC50 values for a single chemical. Multiple assays for a given gene target (e.g. PPARα, PPARδ, PPARγ) are represented by a single symbol, plotted repeatedly. Symbols are the same as in **Figure 3**.

References

1. Varadaraj, J.; Robbins, M. L.; Bock, J.; Pace, S.; MacDonald, D. Dispersion and biodegredation of oil spills on water. http://www.iosc.org/papers/00544.pdf (1 June),
2. ICCVAM ICCVAM Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. http://iccvam.niehs.nih.gov/docs/endo_docs/EDAddendFinal.pdf (15 April),
3. Martin, M. T.; Dix, D. J.; Judson, R. S.; Kavlock, R. J.; Reif, D. M.; Richard, A. M.; Rotroff, D. M.; Romanov, S.; Medvedev, A.; Poltoratskaya, N.; Gambarian, M.; Moeser, M.; Makarov, S. S.; Houck, K. A., Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. *Chem Res Toxicol* **2010,** *23*, (3), 578-90.
4. Romanov, S.; Medvedev, A.; Gambarian, M.; Poltoratskaya, N.; Moeser, M.; Medvedeva, L.; Diatchenko, L.; Makarov, S., Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. *Nat Methods* **2008,** *5*, (3), 253-60.
5. Medvedev A., M. M., Poltoratskaya N., Gambarian M., Medvedeva L., Gambarian M., Martsen E., Romanov S., and Makarov S.S., Global functional profiling of nuclear receptor ligands. **In Preparation**.
6. Judson, R. S.; Houck, K. A.; Kavlock, R. J.; Knudsen, T. B.; Martin, M. T.; Mortensen, H. M.; Reif, D. M.; Rotroff, D. M.; Shah, I.; Richard, A. M.; Dix, D. J., In Vitro Screening of Environmental Chemicals for Targeted Testing Prioritization: The ToxCast Project. *Environ Health Perspect* **2010,** *118*, (4), 485-92.
7. Hemmer, M. J. B., M. G.; Greene, R. M., Comparative Toxicity of Eight Oil Dispersant Products on Standard Aquatic Species. *TBD* **(30 June)**.
8. U.S. EPA OCSPP Harmonized Test Guidelines Series 890 - Endocrine Disruptor Screening Program Test Guidelines. http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series890.htm (11-June),