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

June 30, 2010

U.S. Environmental Protection Agency
Office of Research and Development

Executive Summary

The U.S. Environmental Protection Agency's Office of Research and Development was asked to evaluate the cytotoxicity and potential for interaction with the androgen and estrogen receptors (AR, ER) of eight oil spill dispersants being used, or could be considered for use, in the Gulf of Mexico. These are Corexit 9500 (the current product being used), DISPERSIT SPC 1000, JD 2000, Nokomis 3-F4, Nokomis 3-AA, SAF-RON GOLD, Sea Brat #4, and ZI-400. To address this request, ORD staff and outside collaborators carried out a number of separate studies that were run using *in vitro* (cell-based) assays. A total of 8 cytotoxicity assays, 3 AR agonist assays, 1 AR antagonist assay and 4 ER agonist assays were run on the 8 dispersants, plus reference compounds. Tests were run across a wide range of dispersant concentrations (0.001 to 10,000 parts per million, or ppm). Two dispersants showed a weak signal in one of the four ER assays, but integrating over all of the ER and AR results these data do not indicate that any of the eight dispersants display biologically significant endocrine activity via the androgen or estrogen signaling pathways. All of the dispersants showed cytotoxicity in at least one cell type at concentrations between 10 and 1000 ppm. Both JD 2000 and SAF-RON GOLD tend to be less cytotoxic than the other dispersants. Likewise, DISPERSIT SPC 1000 tends to be more cytotoxic than the other dispersants in the cell-based assays.

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Contents

Contributors

U.S. EPA Office of Research and Development

National Center for Computational Toxicology
David Dix, Keith Houck, Richard Judson, Robert Kavlock, Thomas Knudsen, Stephen Little, Matthew Martin, David Reif, Daniel Rotroff

National Exposure Research Laboratory
Lora Johnson, Mike Hiatt, Tammy Jones-Lepp, John Zimmerman, Andy Grange, Brian Schumacher, Ed Heithmar

National Health and Environmental Effects Research Laboratory
Vickie S. Wilson, Phillip Hartig, L. Earl Gray Jr., Christy Lambright, Mary Cardon, Nicola Wrench, Johnathan Furr, Nathan Jeffay, Barbara Collins, Carol Mitchell

National Institutes of Health

NIH Chemical Genomics Center, Menghang Xia, Srilatha Sakamuru, Ruili Huang, Paul Shinn, Christopher P. Austin

Introduction / Background

The Deepwater Horizon oil spill has led to the use of large amounts of dispersant as part of the integrated approach dealing with the oil spill. Given this fact, questions have arisen about the toxicity of the chemicals used as dispersants themselves. EPA's Office of Research and Development (ORD) was asked to carry out rapid studies to provide information on the potential for toxicity of eight commercially available dispersants. Because some of the dispersants reportedly include nonylphenol ethoxylates (NPEs) that can degrade to isomers of nonylphenol (NP), some of which are proven estrogenic compounds, the potential endocrine effects of the dispersants are of particular concern. For example, NPEs and NPs have been demonstrated to be endocrine disruptors in fish [1]. In response to the request ORD has undertaken a series of short-term *in vitro* studies to determine if any of the dispersants displayed estrogenic, androgenic or other endocrine activity.

ORD developed a strategy to address the questions of endocrine activity and relative toxicity as rapidly as possible. ORD scientists initiated several complementary studies of eight oil spill dispersants being used or considered for use in the Gulf. The issue was to provide some targeted information on the dispersants as quickly as possible. *In vitro* assays are well suited for that purpose. This work complements a study of whole animal toxicity in small fish and brine shrimp also being carried out by ORD. The results of that study are being simultaneously released with this report.

One set of studies used a set of mammalian *in vitro* reporter gene assays in estrogen-responsive and androgen-responsive cells [2, 3] run in-house at ORD laboratories in RTP, NC. Additional studies were conducted by two external labs (NIH Chemical Genomics Center [NCGC] and Attagene Inc.) to run mammalian *in vitro* reporter gene assays to measure androgen and estrogen-response activity. A panel of 74 assays against non-endocrine molecular targets was also included in the Attagene assays. The NCGC and Attagene assays are part of the EPA ToxCast program [4, 5]. All assays evaluated the eight dispersants Corexit® 9500, JD 2000™, DISPERSIT SPC 1000™, Sea Brat #4, Nokomis 3-AA, Nokomis 3-F4, ZI-400 and SAF-RON

GOLD. The performance of the assays was characterized by simultaneously running positive and negative control chemicals. Quantitative cytotoxicity measurements were carried out on each of the cell types used. All data analyses and interpretation were carried out by ORD staff.

It is important to note that positive results *in vitro* only demonstrate that a chemical is a potential endocrine disruptor and that follow-up tests will likely be needed in order to refine or confirm the endocrine activity. For example, effects seen *in vitro* may not be expressed *in vivo*, so additional studies would need to be conducted to verify the *in vitro* results and determine if the potential activity was displayed in whole animals and the dosage levels required to affect organisms.

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Project Goals

1. Determine if any of the eight dispersants displayed estrogenic, androgenic or antiandrogenic activity *in vitro* using a variety of well characterized *in vitro* cell-based assays that utilize different approaches for detecting endocrine driven gene expression changes
2. Determine the dispersant concentration that induced cytotoxicity in multiple cell lines and derive an aggregate measure of cytotoxicity that could be use to rank order the chemicals and to compare with *in vivo* toxicity data obtained in aquatic test species.

Study Summary:

One part of the project was carried out by ORD researchers in partnership with the NIH Chemical Genomics Center [NCGC] and Attagene Inc. Two high throughput assay sets were run on the dispersants, a collection of reference chemicals for ER and AR activity, plus nonylphenol compounds. In addition to assays for AR and ER, this phase of the project produced data on a battery of other transcription factor assays which are part of multiplexed panels including AR and ER assays. Cytotoxicity was evaluated in three cell lines over a range of concentrations.

The other phase of the study was carried out in-house by ORD researchers using multiple assays [2, 3] to measure interaction between the eight dispersants plus reference chemicals and ER or AR. In particular, this work evaluated the eight dispersants for estrogen agonist activity in an estrogen-responsive transcriptional activation assays (ER-TA), for androgen agonist activity in two androgen-responsive transcriptional activation assays (AR-TA), MDA-kb2 and CV-1 assays and for androgen antagonist activity in the MDA-kb2 assay in competition with 1 nM Dihydrotestosterone (DHT). Cytotoxicity was evaluated in each assay at every concentration by both a biochemical assay which assessed metabolic perturbation and by a visual assessment of cytopathic effect on cell viability and morphology.

Chemicals

All assays evaluated eight commercially available oil spill dispersants that were obtained directly from the respective manufacturers. EPA chose these eight dispersants from the dispersants listed on the National Contingency Plan Product Schedule based on three criteria: 1) lower toxicity of the dispersant or of the dispersant when mixed with oil; 2) availability of sufficient quantities to respond to the Gulf spill; and 3) immediate availability of samples for testing. These included Corexit® 9500 (Nalco Inc., Sugarland TX), JD 2000™ (GlobeMark Resources Ltd., Atlanta, GA), DISPERSIT SPC 1000™ (U.S. Polychemical Corp., Chestnut Ridge, NY), Sea Brat #4 (Alabaster Corp., Pasadena, TX), Nokomis 3-AA (Mar-Len Supply, Inc., Hayward, CA), Nokomis 3-F4 (Mar-Len Supply, Inc., Hayward, CA), ZI-400 (Z.I. Chemicals, Los Angeles, CA) and SAF-RON GOLD (Sustainable Environmental Technologies, Inc., Mesa, AZ). All are liquid solutions. Further information on the dispersants, including the limited publicly available information on the composition of dispersants is given in **Appendix A.1**. The oil spill dispersants were tested *in vitro* at concentrations ranging from 0.01 to 1000 ppm in water (vol:vol).

The assays run by NCGC and Attagene included reference compounds recommended for validating ER /AR assays by ICCVAM (Interagency Coordination Committee on the Validation of Alternative Methods)[6] and the U.S. EPA[7]. A preliminary set of reference compounds was obtained from stocks at EPA facilities in RTP NC. Subsequently, additional samples were obtained from Sigma-Aldrich (St. Louis MO). Included in the reference chemicals are both straight chain and branched NP isomers and corresponding example NPEs. The reference chemicals are 17β-Trenbolone (10161-33-8), 17β-Estradiol (50-28-2), Atrazine (1912-24-9), Bisphenol A (80-05-7), Butylbenzyl phthalate (85-68-7), Dibutyl phthalate (84-74-2), Flutamide (13311-84-7), Linuron (330-55-2), 4–Nonylphenol (linear) (104-40-5), p,p' –DDE (72-55-9), p,p'- Methoxychlor (72-43-5), Procymidone (32809-16-8), Vinclozolin (50471-44-8), 2,4,5-T (93-76-5), Bicalutamide (90357-06-5), Cyproterone acetate (427-51-0), Genistein (446-72-0), 4-(tert-octyl), Phenol (140-66-9), 4-Hydroxytamoxifen (68392-35-8), 5α-androstan-17β-ol-3-one (521-18-6) and 4-Nonylphenol, (branched) (84852-15-3). The two nonylphenol ethoxylates are Tergitol NP-9 (127087-87-0) and Igepal CO-210 (68412-54-4). Reference chemicals (powder

form) were solubilized in DMSO to a final concentration of 20 mM. Further information, including lot and batch are given in **Appendix A.2**.

In the in-house ORD assays, a 17β-Estradiol (E2; 50-28-2) dose response was included on every plate in the ER-TA assay as a positive control. 4-Nonylphenol (branched) (84852-15-3; Fluka) and 17α-Trenbolone (Osaka Hayashi Pure Chemical Industries Ltd., CAS no. 80657-17-6, purity 99.9%) were also tested in the estrogen mediated assays. A dihydrotestosterone (DHT; Sigma Chemical; CAS 55206-14-9) dose response was included as a positive control on every plate in the AR-TA assays. The potent androgen, 17α-Trenbolone, was also tested in the androgen agonist assays. Dosing solutions of dispersants and reference compounds were prepared on-site under observation of a Quality Assurance manager. The assays used in the NHEERL assays have been demonstrated [2, 8] to give appropriate responses to known estrogenic or androgenic compounds.

Results

More detailed assay protocols and statistical analysis methods can be found in the Appendices, as well as a Quality Assurance (QA) Statement.

Androgen Receptor Agonist Activity

AR Agonist Assay 1 - Multiplexed reporter transcription unit (RTU) assay

Method Summary: This assay is part of a multiplexed reporter gene panel run by Attagene Inc. (RTP, NC), under contract to the U.S. EPA (Contract Number EP-W-07-049). This assay consists of 48 human transcription factor DNA binding sites transfected into the HepG2 human liver hepatoma cell line as previously described[9]. This *trans* assay employs a mammalian one-hybrid assay consisting of an additional 25 RTU library reporting the activity of nuclear receptor (NR) superfamily members. 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. The *trans* assay evaluates changes in activities of exogenous, chimeric NR-Gal4 proteins. This particular assay evaluated transcription for the Androgen receptor, and uses the code ATG_AR_TRANS. Additional detail of the method is provided in the **Appendix B.1**. Concentration-response titration points for each compound were fitted as described in **Appendix C.** For this analysis, there were either 4 replicates in 16 concentrations, except for SAF-RON GOLD which was only tested in 2 replicates and 8 concentrations.

Results: No activity was seen for any of the dispersants

AR Agonist Assay 2 - AR beta-lactamase Assay

Method Summary: This assay was run at the NIH Chemical Genomics Center (NCGC; Rockville, MD) in collaboration with EPA as part of the Tox21 collaboration[10]. A beta-lactamase reporter-gene cell-based assay [GeneBLAzer® AR-UAS-bla-GripTite™ assay developed by Invitrogen] was used to measure AR ligand 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). Additional detail of the method is provided in the **Appendix B.2**. Concentration-response titration points for each compound were fitted as described in **Appendix C**. For this analysis, there were 8-10 replicates in 24 concentrations.

Results: The only dispersant that showed any activity in any of the AR assays was JD 2000, which was active in both the NCGC ER and AR agonist and antagonist assays in all runs with AC50 values ranging from 100-270 ppm (AR) and 82-120 ppm (ER). There was no apparent cytotoxicity in any of the cell line for JD 2000 (see results below). The EMax values for JD 2000 in all of these assays were significantly greater than the values for positive control chemicals, and in the antagonist assays, this dispersant looked like a "super-activator" rather than an antagonist. All of this data taken together indicates strongly that some non-specific activation is occurring that is independent of ER or AR. We have found previously that compounds identified as promiscuous "super-activators" in multiple beta-lactamase reporter gene assays with a narrow potency range (a <3-fold difference in potency is within the experimental variations of these assays) are mostly auto fluorescent (R Huang, unpublished data). Thus, the activity observed for JD 2000 is likely an artifact of the beta-lactamase assay format. Preliminary results from three additional beta-lactamase assays for non-steroid receptor targets all showed the JD 2000 "super-

activation". Considering the totality of the data, we conclude that JD 2000 does not exhibit ER or AR transactivation activity. To further confirm that this JD 2000 activity is non-specific and not due to ER or AR activation, we are running several follow-up assays with NCGC: known antiestrogens and antiandrogens are being used to show that JD 2000 activity is not suppressed; and we will complete our analysis of results for the the three non-steroid receptor beta-lactamase assays are being run with JD 2000 to show that this non-specific activity occurs independent of ER and AR.

AR Agonist Assay 3 - MDA-kb2 Androgen-responsive transcriptional activation assay

Method Summary: This assay, run in-house by NHEERL researchers, utilized MDA-kb2 cells[2]. These cells contain endogenous human androgen receptor capable of inducing transcription of an androgen responsive gene (AR-TA). This assay employs a luciferase gene driven by the androgen responsive MMTV promoter which has been stably integrated into the cells. When androgen mimicking compounds (i.e. compounds that act as androgen agonists) are present, these cells produce luciferase in a concentration proportionate to the efficacy of the androgen mimic. Nine concentrations of each dispersant were tested for agonist activity. Each concentration was evaluated in a total of eight replicates (two independent evaluations with four replicates per assay). The first dilution of each sample was a 1:100 dilution (i.e. 0.01 dilution or 10,000 ppm) of the dispersant in cell culture medium followed by eight additional 10-fold serial dilutions. Additional detail of the method is provided in the **Appendix F**.

Results: The ability of the eight dispersants to stimulate luciferase expression in this cell line was compared to DHT. The DHT positive control dose induced luciferase expression in MDA Kb2 cells in a precise and reproducible manner within and among the plates (**Figure 1**. DHT and 17 α-Trenbolone data in MDA Kb2 cells). None of the eight dispersants displayed any potential androgenicity (i.e. did not simulate luciferase induction) at any concentration in the MDA Kb2 cell line (**Figure 2** dispersant results in MDA Kb2 cells). In fact, all the dispersants displayed significantly reduced luciferase levels due to cytotoxicity at high dispersant concentrations. The synthetic androgen 17α-Trenbolone acted as a full androgen agonist at relatively low concentrations.

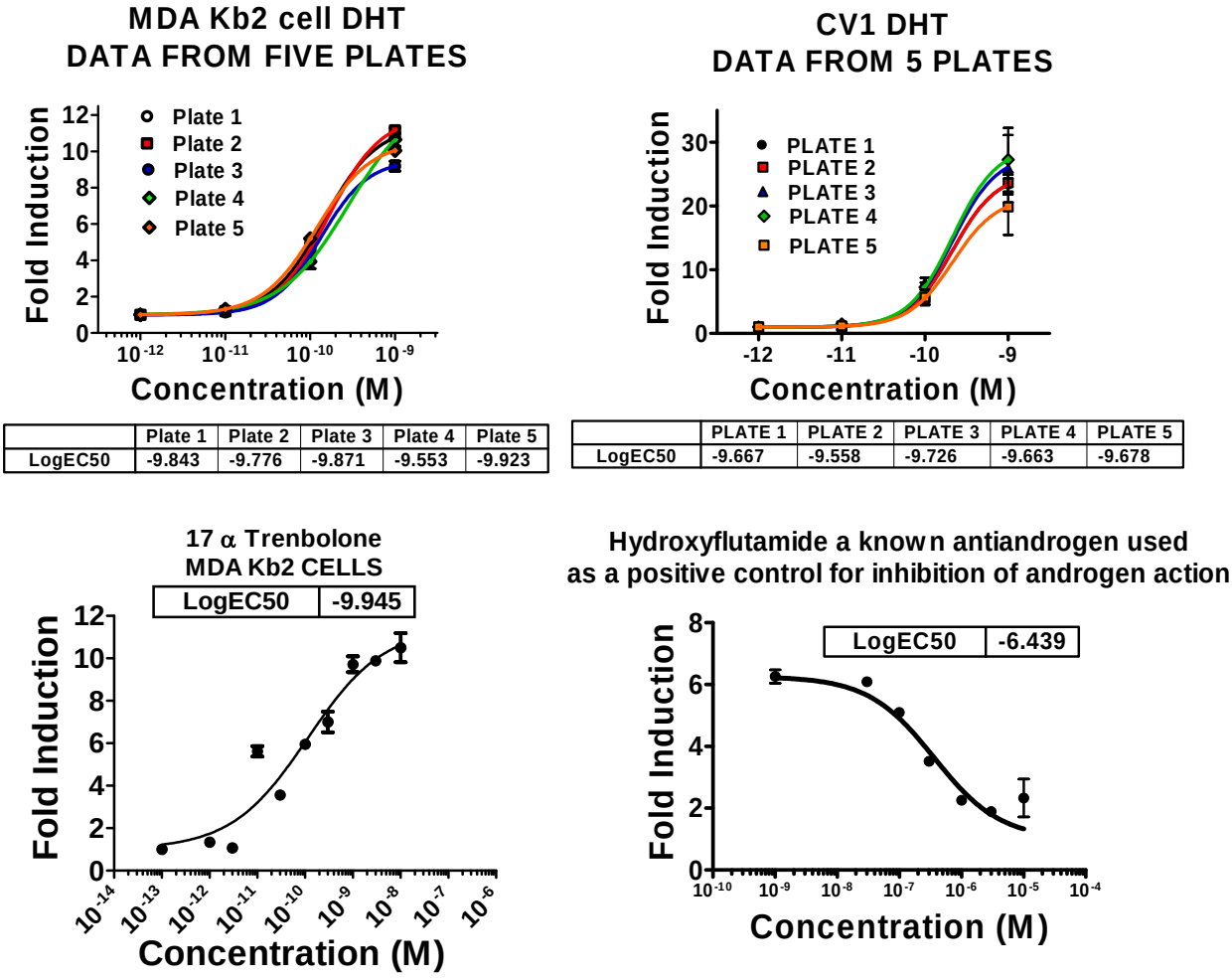


	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
LogEC50	-9.843	-9.776	-9.871	-9.553	-9.923

	PLATE 1	PLATE 2	PLATE 3	PLATE 4	PLATE 5
LogEC50	-9.667	-9.558	-9.726	-9.663	-9.678

Figure 1: Included are, nonlinear regression plots of the effects of the reference androgen dihydrotestosterone (DHT) in two androgen sensitive cell lines (MDA Kb2 upper left and CV1 upper right), stimulatory effects of the synthetic androgen found in some aquatic systems in MDA Kb2 cells (lower left) and antagonism of the 1 nM DHT by the antiandrogenic drug hydroxyflutamide in MDA Kb2 cells. Data are expressed as fold over the media plus the ethanol control value. The X axis is in log scale. Values are means plus or minus standard errors of the mean.

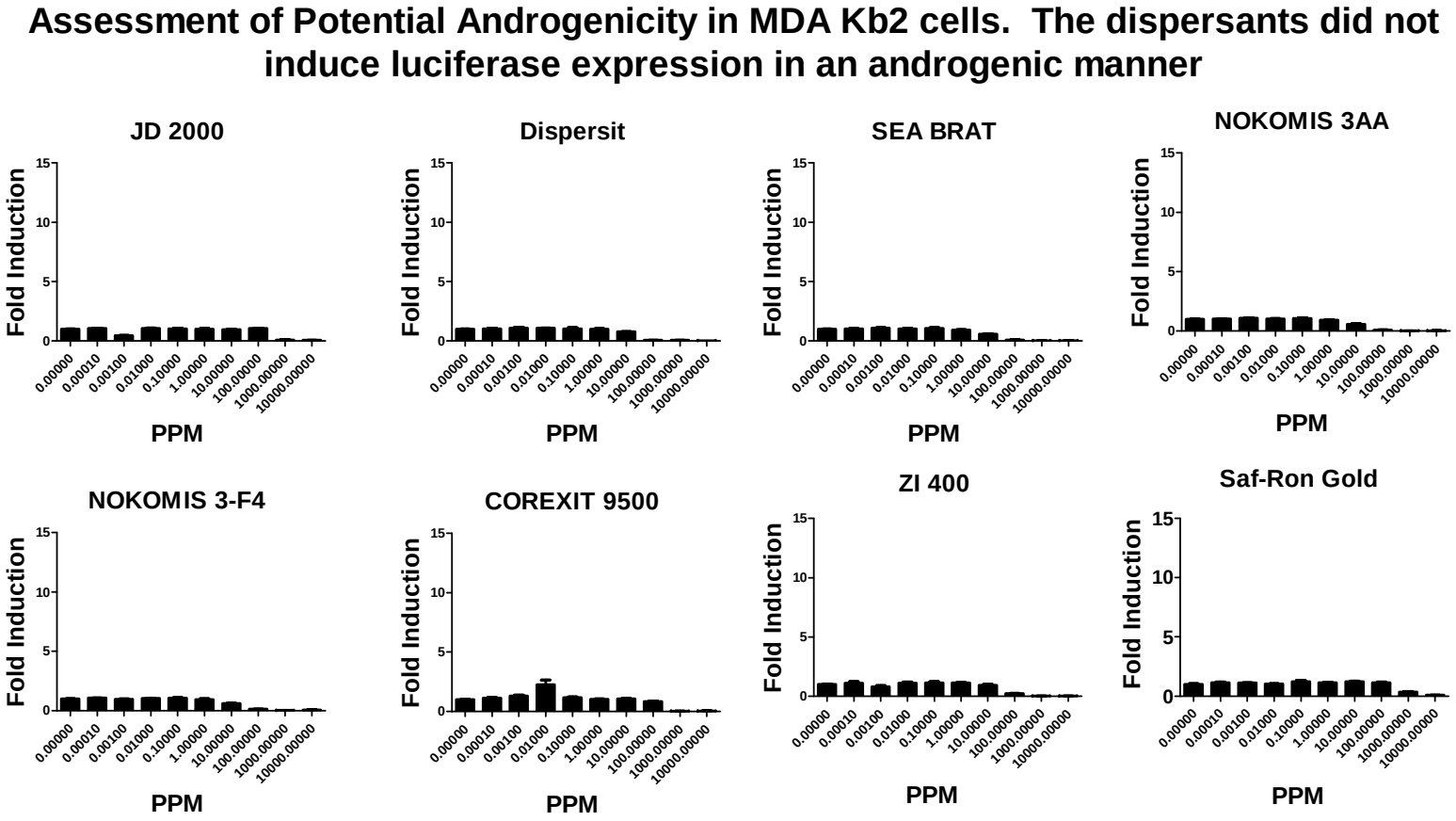


Figure 2: Assessment of the potential androgenic activity of the eight dispersants in MDA Kb2 cells. Data are expressed as fold over the media plus ethanol vehicle control. Values are means plus or minus standard errors of the mean. Dispersants did not stimulate luciferase induction over the control fold value (control fold =1).

AR Agonist Assay 4 - CV-1 transient transcription assay

Method Summary: This assay run in-house by NHEERL is similar to the MDA-kb2 in that it also assesses the ability of a compound to mimic an androgen. This assay, however, uses CV-1 cells which do not express either endogenous androgen or estrogen receptors. In contrast to the MDA-kb2 assay, both the androgen receptor and the androgen responsive MMTV promoter- luciferase reporter constructs are introduced into the CV-1 cells for each assay via transient transfection. Nine concentrations of each dispersant were tested for agonist activity in both AR-TA assays. Each concentration was tested in quadruplicate. The first dilution of each sample was a 1:100 dilution (i.e. 0.01 dilution or 10,000 ppm) of the dispersant in cell culture medium followed by eight additional 10-fold serial dilutions. Method details are provided in the **Appendix F**.

Results: Similar to the results of the MDA-kb assays, DHT induced precise and reproducible effects on luciferase expression within and among the plates (Figure 1) and none of the eight dispersants displayed any potential androgenicity (i.e. did not simulate luciferase induction) at any concentration in the CV-1 assay (**Figure 3**). In fact, all the dispersants significantly reduced luciferase level due to cytotoxicity at high concentrations**.**

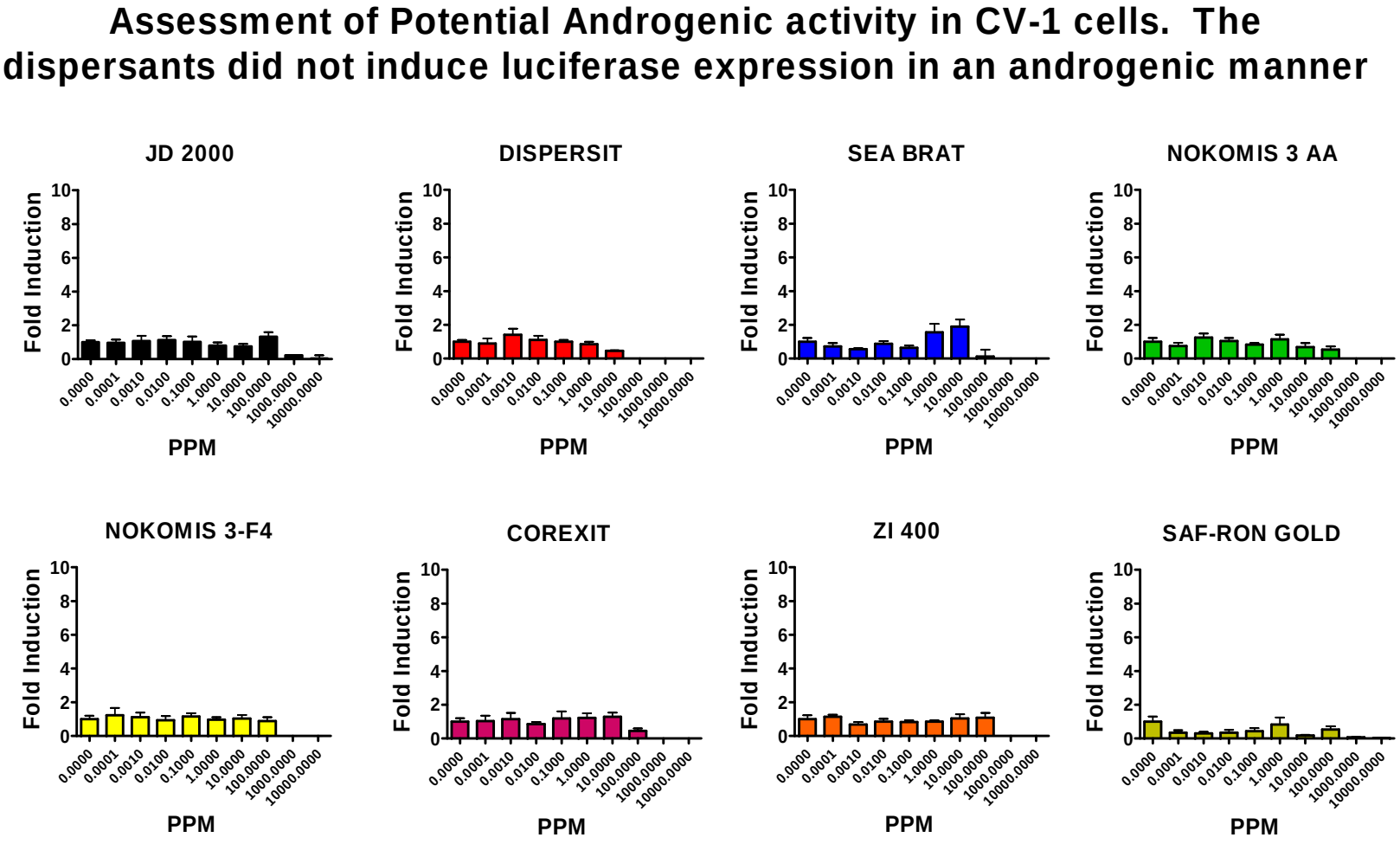


Figure 3: Assessment of the potential androgenic activity of the eight dispersants in CV-1 cells. Data are expressed as fold over the media plus ethanol vehicle control. Values are means plus or minus standard errors of the mean. Dispersants did not stimulate luciferase induction over the control fold value (control fold =1).

Androgen Receptor Antagonist Activity

AR Antagonist Assay 1 - MDA-kb2 Androgen-responsive transcriptional activation assay in antagonist mode

Method Summary: The eight dispersants were also evaluated for antagonist activity in the MDA-kb2 cell line, run in-house by NHEERL researchers, by testing each dispersant in the presence of a near maximally stimulating concentration dihydrotestosterone (1 nM DHT). In the presence of an anti-androgen, the luciferase activity induced by DHT would be reduced proportionally to the concentration of the anti-androgen. A DHT concentration-response curve was included on each 96-well plate with the dispersants. The well-characterized antiandrogen hydroxyflutamide (CAS 80657-17-6) was run as a positive control (**Figure 1**). Six concentrations of each dispersant ranging from 0.0001 ppm to 10 ppm were tested for antagonist activity. Higher concentrations were not evaluated due to cytotoxicity seen in both MTT and CPE assays (discussed later in this document). Each concentration was tested in quadruplicate. Additional detail of the method is provided in the **Appendix F**.

Results: None of the eight dispersants displayed any potential antiandrogenicity (i.e. did not inhibit DHT-induced luciferase induction) at concentrations below 10 ppm (1E-5 dilution). At 10 ppm several of the dispersants reduced DHT induced luciferase activity, but the effects were significant (by ANOVA followed by a post hoc Dunnett's test) for the dispersants SPC 1000 and Nokomis 3-AA (**Figure 4**). As shown in **Figure 5a**, these two dispersants were the most toxic of the dispersants to MDA Kb2 cells so it is extremely unlikely that these effects represent competitive inhibition of DHT binding to the ligand binding domain of the androgen receptor. In contrast, hydroxyflutamide, used as a positive control, completely inhibited androgen-induced luciferase induction at concentrations about 1000 fold higher than of the concentration of DHT used in this assay.

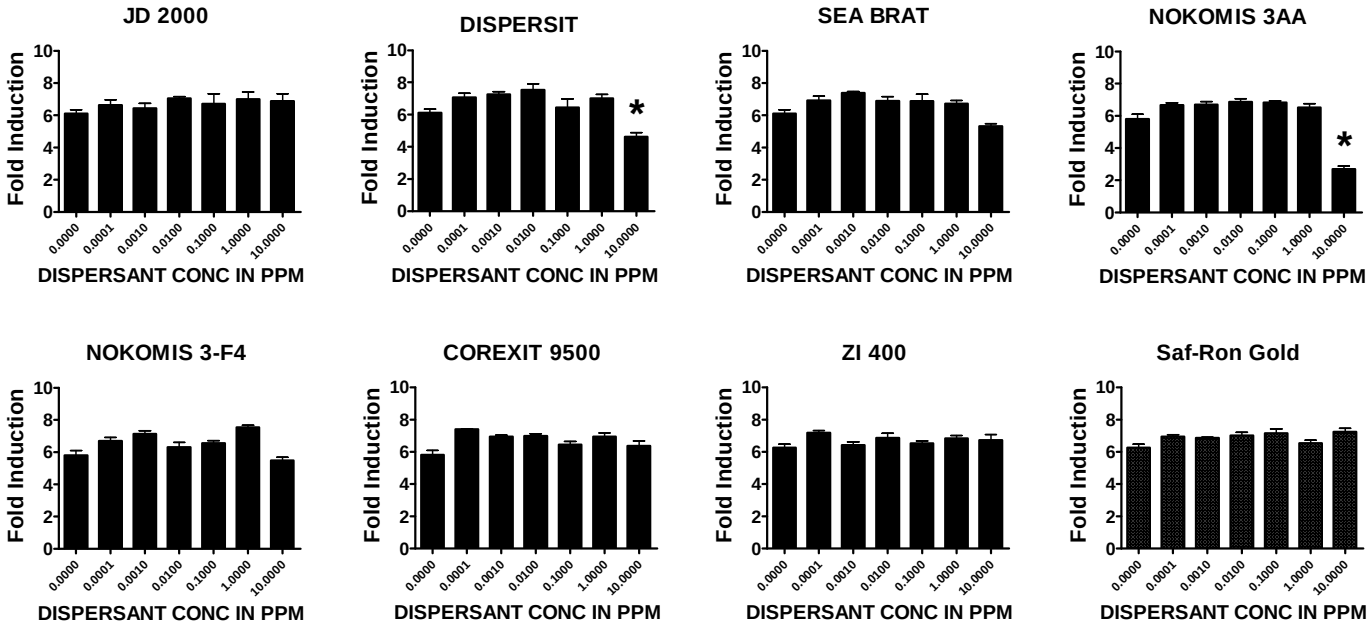
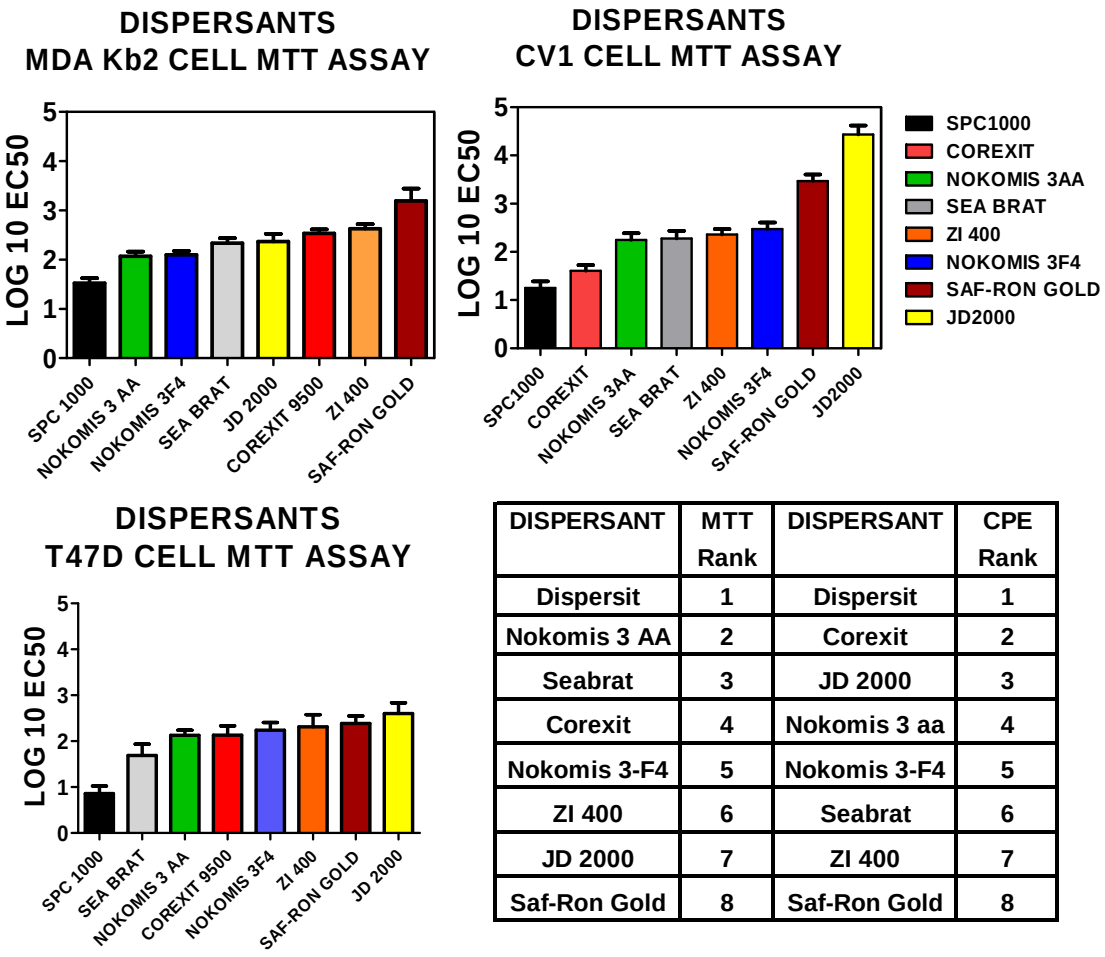


Figure 4: Assessment of the potential antiandrogenic activity of the eight dispersants in MDA Kb2 cells. Data are expressed as fold over the media plus ethanol vehicle control. Values are means plus or minus standard errors of the mean. DISPERSIT SPC 1000 and Nokomis 3-AA were the only dispersants that significantly reduced DHT-stimulated luciferase induction (DHT control fold about 6) an effect which we interpreted to result from cytotoxicity at 10 ppm, the highest concentration used in this assay.



DISPERSANT	MTT Rank	DISPERSANT	CPE Rank
Dispersit	1	Dispersit	1
Nokomis 3 AA	2	Corexit	2
Seabrat	3	JD 2000	3
Corexit	4	Nokomis 3 aa	4
Nokomis 3-F4	5	Nokomis 3-F4	5
ZI 400	6	Seabrat	6
JD 2000	7	ZI 400	7
Saf-Ron Gold	8	Saf-Ron Gold	8

Figure 5: Summary of the Cytotoxic effects of the eight dispersants in the MTT and visual cytopathic examinations (CPE) in three cell lines. The histograms display the EC50 values for a reduction in MTT levels (determined by nonlinear regression on Prism 5.0) for the MDA Kb2 (upper right panel), CV1 (upper right panel) and T47D Kbluc

(lower left panel) cells. In the table in the lower right panel, the overall potency of the dispersants in the MTT and CPE assays is shown, with a ranking of 1 being the most potent in inducing cytotoxicity and 8 being the least cytotoxic dispersant. DISPERSIT SPC 1000 was ranked as the most cytotoxic by both methods and SAF-RON GOLD is the least toxic of the eight dispersants. (The appendices contain additional details on this and the EC50 values are compared using a multiple range test).

Estrogen Receptor Agonist Activity

ER Agonist Assay 1 - Multiplexed reporter transcription unit (RTU) *trans* assay

Method Summary: This assay is part of a multiplexed reporter gene panel run by Attagene Inc. (RTP, NC), under contract to the U.S. EPA (Contract Number EP-W-07-049). This assay consists of 48 human transcription factor DNA binding sites transfected into the HepG2 human liver hepatoma cell line as previously described[9]. This *trans* assay employs a mammalian one-hybrid assay consisting of an additional 25 RTU library reporting the activity of nuclear receptor (NR) superfamily members. 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. The *trans* assay evaluates changes in activities of exogenous, chimeric NR-Gal4 proteins. This particular assay evaluated transcription for the Estrogen receptor alpha, and uses the code ATG_ERa_TRANS. This assay was run in twice in separate weeks, and in each case, run in duplicate. For this analysis, there were either 4 replicates in i6 concentrations, except for SAF-RON GOLD which was only tested in 2 replicates and 8 concentrations. Additional detail of the method is provided in the **Appendix B.1**. Concentration-response titration points for each compound were fitted as described in **Appendix C.**

Results: We observed statistically significant ER activity in two of the dispersants in the Attagene *trans*-ERα assay (Nokomis 3-F4 and ZI-400), detailed in **Table 1**. **Figure 6** (bottom panels) shows the concentration-response curves for the two active dispersants, which have EMax (maximum efficacy) values of between 3 and 4. This is in contrast to 17β-Estradiol (top left panel, blue curve), which has an EMax value of 20. The top right panel of the figure shows the corresponding reference curve for the *cis*-ERE assay, showing that 17β-Estradiol only elicits a response about half of that seen in the *trans* assay. To help interpret these results, we

simultaneously analyzed their performance on a set of 19 reference chemicals recommended by ICCVAM[6] and EPA OPPT[7]. This analysis (detailed in **Appendix E**) shows that these assays perform well for both positive and negative predictive value. The *trans*-ERα assay correctly matched ICCVAM expectation for 15 of 17 reference chemicals, with one false positive and one false negative. A comparison of the *cis* and *trans* assays shows that the reference chemicals in the *cis* assay consistently produce EMax values about half of that seen in the trans assay. This would explain the absence of observable activity for these dispersants in the *cis* assay, because we do not consider curves with EMax values below 2. The other curves in the bottom panels of **Figure 6** show data for NP and NPE compounds, described below.

Chemical	**AC50 (ppm)**	**EMax**	**R^2**	**p-value**
Nokomis 3-F4	16	3.9	0.65	0.00017
ZI-400	25	3.4	0.68	0.0041

Table 1: Summary results for the Attagene *trans*-ERα assay for the positive dispersants. EMax: maximal fold change. AC50: concentration at which 50 of maximal activity is seen.

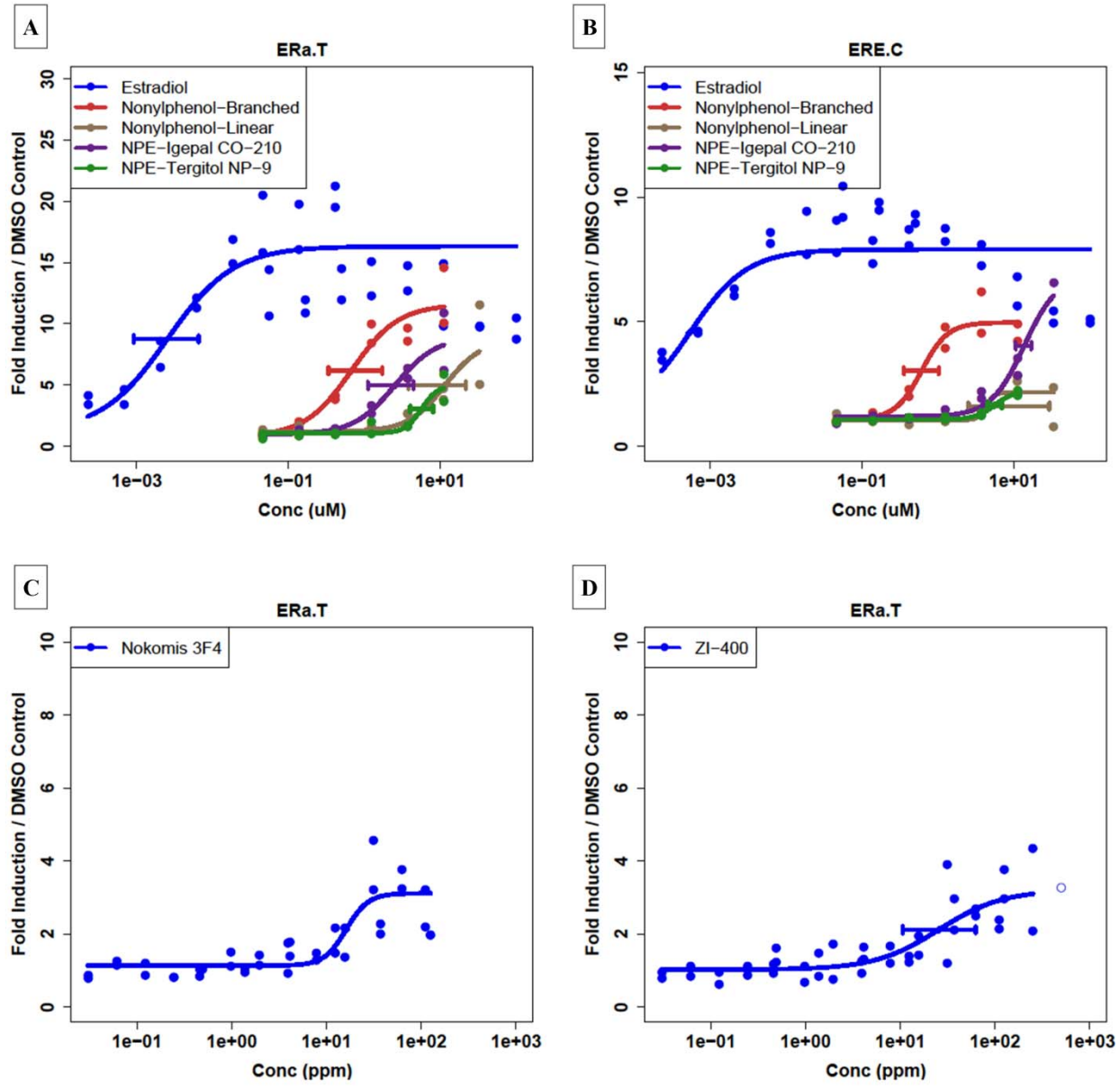


Figure 6: Concentration-response curves for the E2, NP and NPE compounds, and the two dispersants showing activity in Attagene *trans*-ERα assay. Top: E2 and the 4 NP / NPE compounds in the Attagene *trans*-ERα assay (left) and the Attagene *cis*-ERE assay (right). Bottom: ZI-400 and Nokomis 3-F4 in the Attagene *trans*-ERα assay. For the nonylphenol compounds, only two replicates were run.

Nonylphenol-related activity: It is known that some of the dispersants contain NPEs. Our initial hypothesis was that any estrogenic activity detected for the complex mixtures could be due to the

NPEs or to NP itself generated by *in situ* degradation of the NPE, or residual contamination from synthesis of the NPE. Consequently, we tested two nonylphenols (one linear and one branched, technical grade) and two commercial NPEs in the Attagene assays. **Table 2** shows the results of this analysis, and **Figure 6** shows the corresponding dose-response curves for the Attagene ER assays. From these data, one can see that these cell-based assays show ER activity for both the NPs and the NPEs. The branched, technical grade NP is the most potent, but the second most potent is the NPE Igepal CO-210. These data indicates that the presence of an NP or NPE in a mixture could give rise to ER activity such as was seen for the dispersants Nokomis 3-F4 and ZI-400. Public information (given in **Appendix A**) indicates that ZI-400 does in fact contain an NPE.

Chemical	**Assay**	**AC50 (μM)**	**R^2**	**EMax**	**p-value**
4-Nonylphenol (linear) 104−40−5	*trans* -ERα	11	0.77	8.3	0.29
	cis-ERE	4.3	0.55	2.7	0.096
4-Nonylphenol (branched) 84852−15−3	*trans* -ERα	0.68	0.91	12	0.0049
	cis-ERE	0.61	0.092	5.4	4.9E-5
Tergitol NP-9 127087−87−0	*trans* -ERα	5.7	0.86	4.8	0.18
	cis-ERE	5.6	0.96	2.1	0.042
Igepal CO−210 68412−54−4	*trans* -ERα	2.5	0.89	8.5	0.19
	cis-ERE	14	0.96	6.5	2.1E-11

Table 2: Results of ER assays on NPs and NPEs.

To summarize this section, estrogen receptor (ER) activity was observed in two of the dispersants in the Attagene *trans*-ERα assay (ZI-400 and Nokomis 3-F4), although at relatively high concentrations and with low efficacy (EMax). We have also shown that NPs and NPEs are also active in the *trans*-ERα assay. Therefore, the activity in ZI-400 and Nokomis 3-F4 is suggestive of the presence of an NP or NPE as part of the mixture. We know that this is the case with ZI-400. The ER effect seen for these dispersants is weak, which is also suggestive of there being only a relatively small amount of NPE or some other estrogenic substance in the total mixture.

ER Agonist Assay 2 - Multiplexed reporter transcription unit (RTU) *cis* assay

Method Summary: This assay is part of a multiplexed reporter gene panel run by Attagene Inc. (RTP, NC), under contract to the U.S. EPA (Contract Number EP-W-07-049). This assay consists of 48 human transcription factor DNA binding sites transfected into the HepG2 human liver hepatoma cell line as previously described[9]. A major difference between the *cis* and *trans* system is that in *cis* activities of endogenous transcription factors are measured. This particular assay evaluated transcription for the Estrogen receptor element (ERE), and uses the code ATG_ERE_CIS. For this analysis, there were either 4 replicates in i6 concentrations, except for SAF-RON GOLD which was only tested in 2 replicates and 8 concentrations. Additional detail of the method is provided in the **Appendix B.1**. Concentration-response titration points for each compound were fitted as described in **Appendix C.**

Results: No statistically significant activity was seen for any of the dispersants

ER Agonist Assay 3 – ER-alpha beta-lactamase Assay

Method Summary: This assay was run at the NIH Chemical Genomics Center (NCGC; Rockville, MD) in collaboration with EPA as part of the Tox21 collaboration[10]. A beta-lactamase reporter-gene cell-based assay [ERα-UAS-bla GripTite™ 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

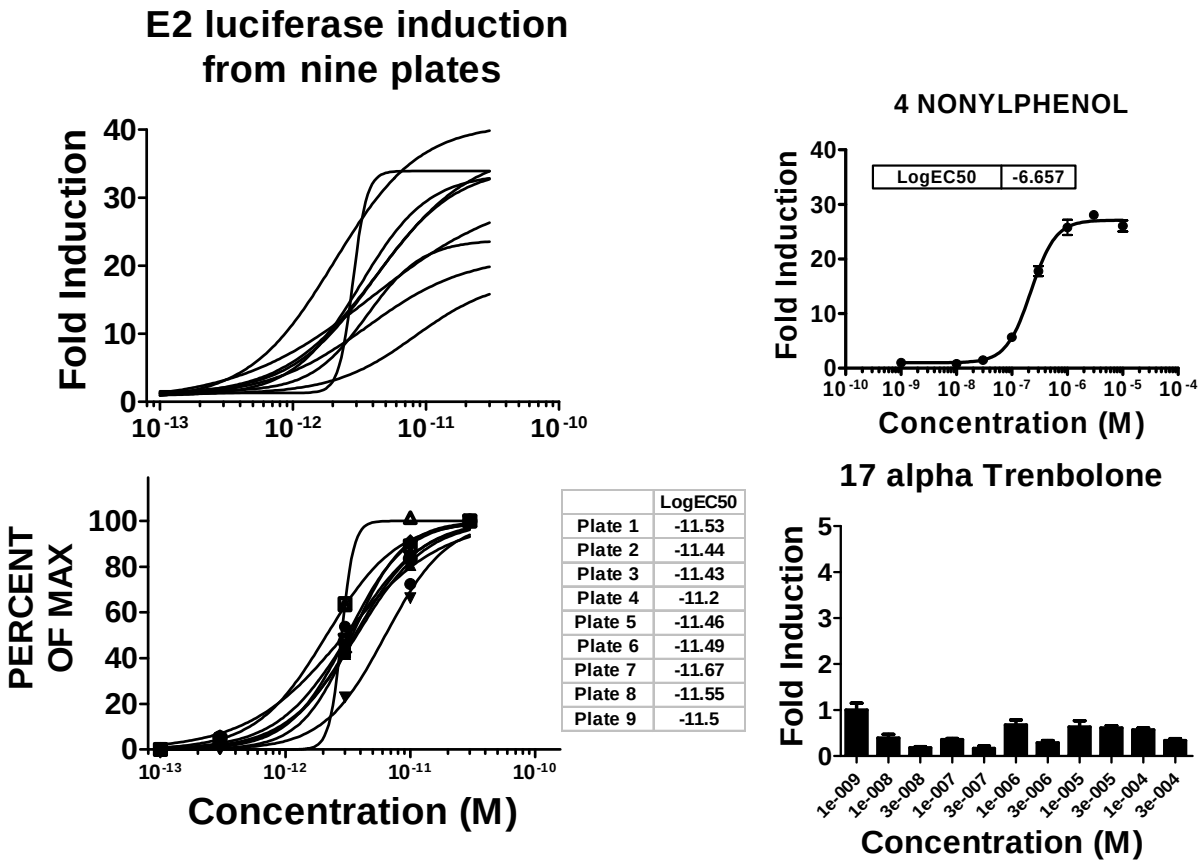
each compound were fitted to the Hill equation yielding concentrations of half-maximal stimulation (EC_{50}), half-maximal inhibition (IC_{50}) and maximal response (efficacy) values. For this analysis, there were 8-10 replicates in 24 concentrations. Additional detail of the method is provided in the **Appendix B.3**. Concentration-response titration points for each compound were fitted as described in **Appendix C.**

Results: No biologically relevant results were seen for any of the dispersants. See the description above under the corresponding AR assay for JD 2000.

ER Agonist Assay 4 - T47D-KBluc estrogen-responsive transcriptional activation assay

Method Summary: T47D-KBluc, is an estrogen receptor-mediated transcriptional activation assay (ER-TA) that detects the ability of chemicals to mimic estrogen[8]. This assay was run in-house by NHEERL researchers. The cells contain endogenous human estrogen receptors alpha and beta and are stably integrated with an engineered luciferase reporter gene controlled by triplet estrogen response elements. When the cells are exposed to hormone mimics, the mimicking chemical binds the estrogen receptor and activates production of the luciferase reporter gene. The luciferase product is measured in a light emitting reaction. Additional detail of the method is provided in the **Appendix F**.

Results: The ability of the eight dispersants to stimulate luciferase expression in this cell line was compared to 17β-Estradiol (CAS 50-28-2: a concentration-response curve to E2 was included on each 96 well plate with the dispersants) and to 4-Nonylphenol (branched) (CAS 84852-15-3) (**Figure 7 a,b**). 17α-Trenbolone (CAS 80657-17-6) was run as a negative control herein (**Figure 7d**) and as a positive control in the assessment of androgenicity. None of the eight dispersants displayed any potential estrogenicity (i.e. did not simulate luciferase induction) at any concentration in the current investigation (**Figure 8**). In fact, all the dispersants significantly reduced luciferase levels at high concentrations due to cytotoxicity.



	LogEC50
Plate 1	-11.53
Plate 2	-11.44
Plate 3	-11.43
Plate 4	-11.2
Plate 5	-11.46
Plate 6	-11.49
Plate 7	-11.67
Plate 8	-11.55
Plate 9	-11.5

Figure 7: Estradiol 17β-induced (E2) luciferase expression in the nine plates used in the current study with T47D Kbluc cells, expressed as fold over media plus ethanol vehicle control (upper left) and percent of the maximal E2 stimulation (lower left). The

effects of the xenoestrogen 4-Nonlyphenol (Branched) are shown in the upper right and the lack of estrogenicity of the synthetic androgen 17α-Trenbolone are shown in the lower right panels. Values are means plus or minus standard errors of the mean.

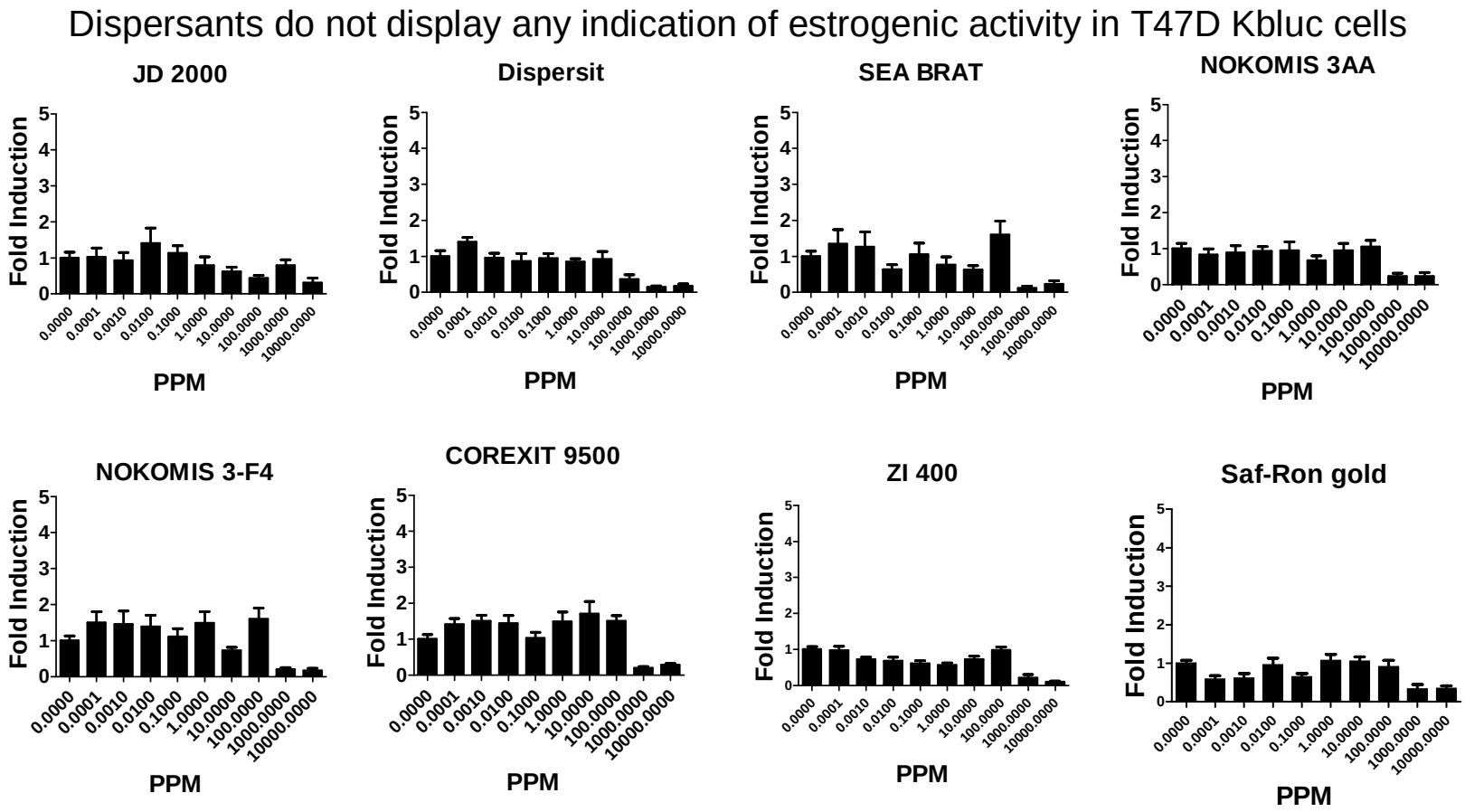


Figure 8: Assessment of the potential estrogenic activity of the eight dispersants in T47D Kbluc cells. Data are expressed as fold over the media plus ethanol vehicle control. Values are means plus or minus standard errors of the mean. Dispersants did not stimulate luciferase induction over the control fold value (control fold =1).

Cytotoxicity

Cytotoxicity Assay 1 –HepG2 Cells

Method Summary: Dispersants were tested for cytotoxicity against HepG2 cells in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay (15) following 24 h chemical exposure to 16 concentrations with an upper concentration of 1000 ppm. All concentrations were run in triplicate. This assay was run by Attagene Inc. LC50 values were determined by fitting curves as described in **Appendix C**. Results of cytotoxicity assessment are shown below. For this analysis, there were either 4 replicates in 16 concentrations, except for SAF-RON GOLD which was only tested in 2 replicates and 8 concentrations.

Cytotoxicity Assay 2 –AR *bla* Cells

Method Summary: Cell viability after compound treatment was measured in these AR *bla* cells using a luciferase-coupled ATP quantitation assay (CellTiter-Glo viability assay, Promega). This assay was run by the NIH Chemical Genomics Center. 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%). LC50 values were determined by fitting curves as described in **Appendix C**. Results of cytotoxicity assessment are shown below. For this analysis, there were 8-10 replicates in 24 concentrations.

Cytotoxicity Assay 3 –ER *bla* Cells

Method Summary: Cell viability after compound treatment was measured in these ER *bla* cells using a luciferase-coupled ATP quantitation assay (CellTiter-Glo viability assay, Promega). This assay was run by the NIH Chemical Genomics Center. 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%). LC50 values were determined by fitting curves as described in **Appendix C.** Results of cytotoxicity assessment are shown below. For this analysis, there were 8-10 replicates in 24 concentrations.

Cytotoxicity Results (Assays 1-3)

Results of Cytotoxicity Assays 1-3 are summarized below the description of Assays 4-6.

Cytotoxicity Assays 4 THRU 9: measurements in T47D-KBluc, MDA-kb2, and CV-1 cells (MTT and CPE assessments) (5 independent assessments).

Methods summary: The ability of the dispersants to produce a general toxic effect on each of the cell lines used in the in the NHHERL in-house assays was assessed by both observational and biochemical methods. First, each well of cells in every assay was evaluated by visual microscopic examination utilizing a five point cytopathic effect (CPE) criteria scale ranging from 0 (no visual toxicity) to 4 (total cell death). CPE assessment criteria were as follows: 0 = no observed effect; 1= subtle changes suggesting effect; 2 = definite effects or death in a at least 25% of cells; 3 = 50 to 75% of cells effected; 4 = 100% of cells effected/cell death.

Second, an assessment of the metabolic perturbation of cell health was quantitated by monitoring the ability of cells to metabolize 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)[11]. In this biochemical assay, healthy cells are capable of converting a yellow MTT solution into a blue dye. The healthier the cell the more blue dye produced. This biochemical assay is an indicator that the cells are metabolically active and is a measurement of general cell health. The MTT assay is a quantitative evaluation of mitochondrial function of the cells whereas the first method was a qualitative microscopic cytopathological evaluation (CPE) of cell viability and morphology

Cytotoxicity Results (Assays 4-9)

All eight dispersants disrupted cell function and caused cell death in all three cell lines in the two highest concentrations (0.01 and 0.001, or 10,000 and 1,000 ppm, respectively). Furthermore, none of the dispersants produced any sign of cytotoxicity at concentrations below 1 ppm (**Figures 9-12**).

Cytopathological evaluation of dispersant cytotoxicity (eight plates/dispersant, four replicate wells/plate/conc) in MDAKb2, CV 1 and T47D Kbluc cells. Figures are ranked from top left to bottom right in cytotoxic potency at 1, 10 and 100 ppm. At higher concentrations CPE scores were 3-4 for all dispersants and no CPE was observed at concentrations below 1 ppm. The maximum total CPE score is 12, 4 per assay

	cytopathic effect: defined
no CPE	none observed
CPE +1	subtle changes suggesting effect
CPE+2	definite changes in more than 25 % of cells
CPE+3	50-75 % of cells effected
CPE+4	100% cells effected/dead

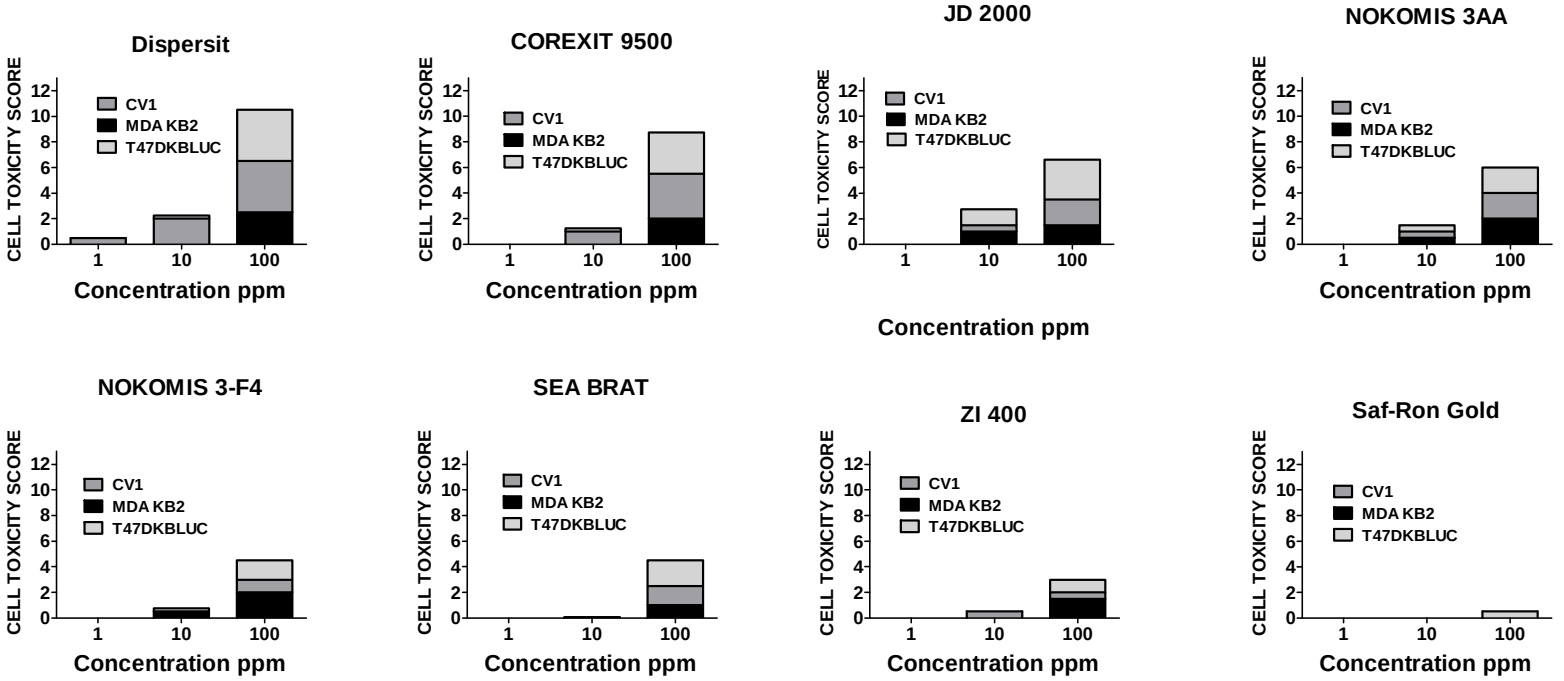


Figure 9:

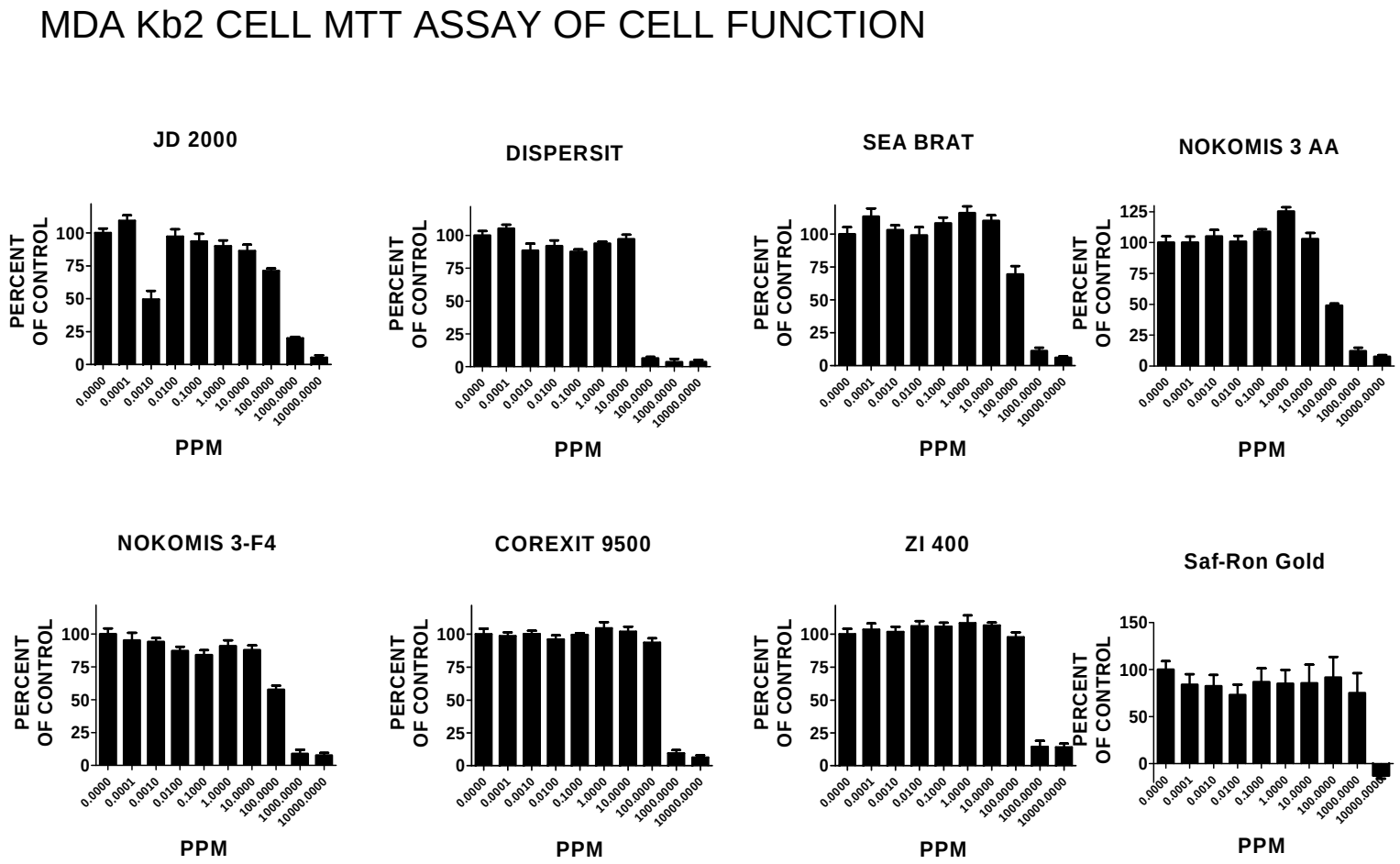


Figure 10: Toxic effects of the eight dispersants on MMT levels in MDA Kb2 cells. A reduction in MTT levels is an indicator of cytotoxcity, seen with all dispersants at the two higher concentrations (1000 and 10,000 ppm) and at 100 ppm with several of the dispersants. Data are expressed as percent of control (media).

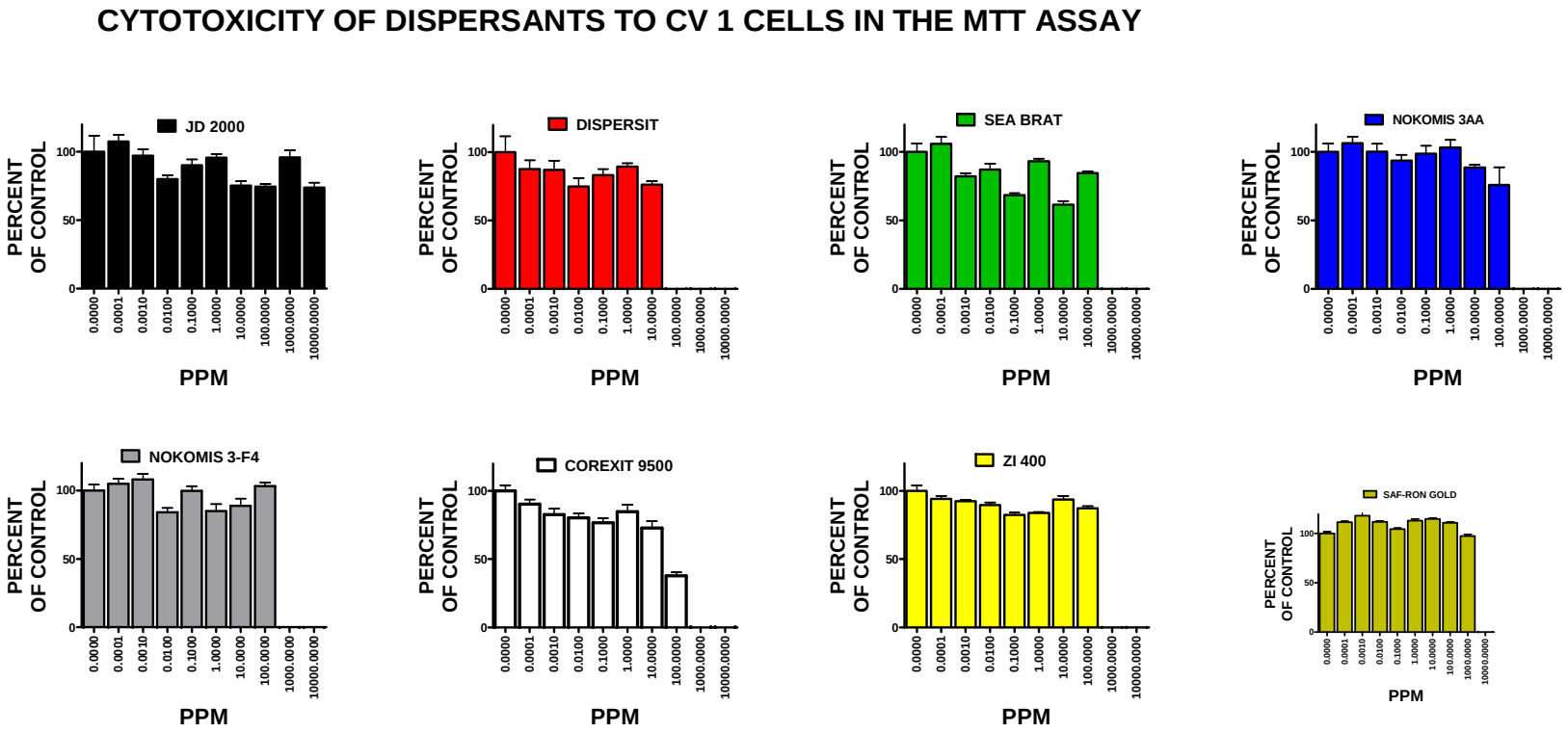


Figure 11: Toxic effects of the eight dispersants on MMT levels in CV-1 cells. A reduction in MTT levels is an indicator of cytotoxcity, seen with all dispersants at the two higher concentrations (1000 and 10,000 ppm) except JD 2000 (no toxicity was seen at any concentration) and SAF-RON GOLD (toxicity was seen only at the highest concentration of 10,000 ppm). DISPERSIT SPC 1000 and Corexit also induced cytotoxicity at 100. Data are expressed as percent of control (media).

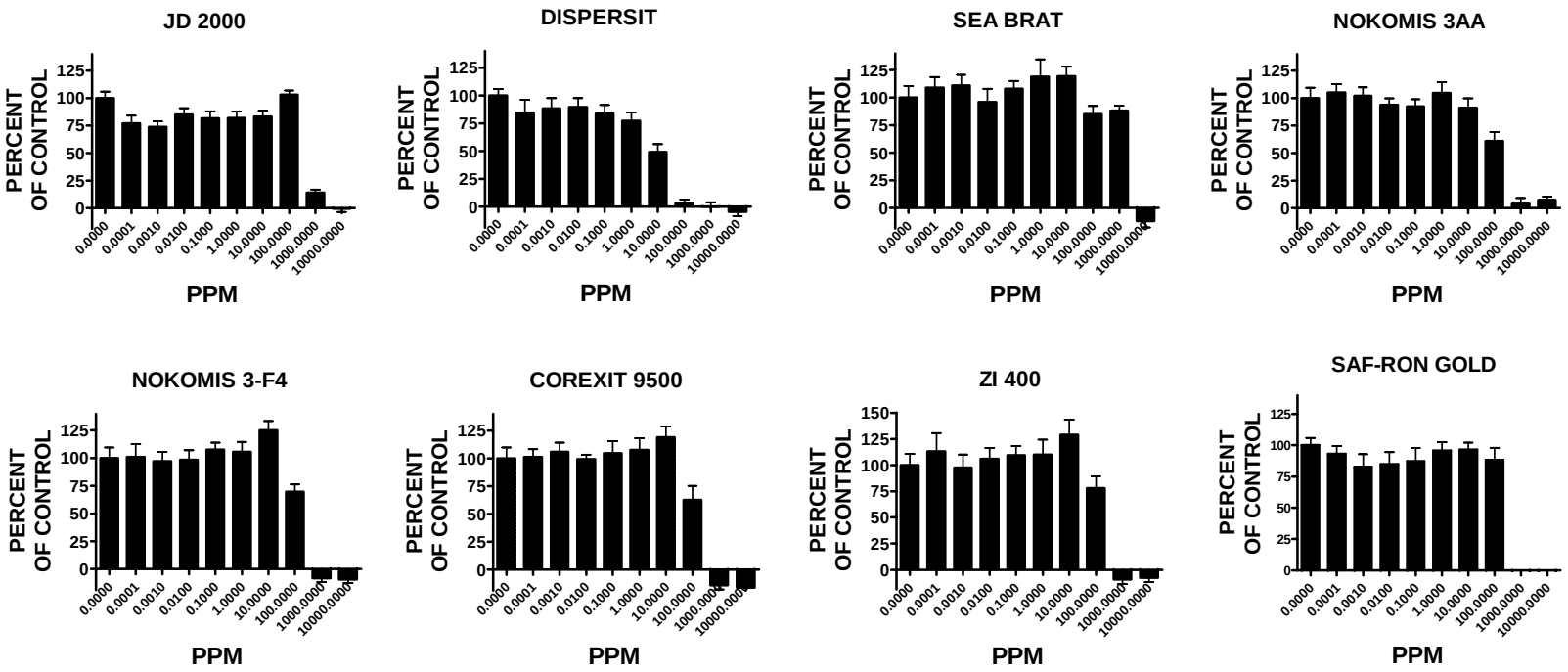


Figure 12: Toxic effects of the eight dispersants on T47D Kbluc cells. A reduction in MTT levels is an indicator of cytotoxicity, seen with all dispersants at the two higher concentrations (1000 and 10,000 ppm) and at 100 ppm with several of the dispersants. Data are expressed as percent of control (media).

The lowest observed effective concentration (LOEC) for dispersant-induced reductions in MTT, estrogen, androgen and antiandrogen assays are reported in **Table 3**. In the table, the noted changes in the two androgen and the estrogen agonist assays do not result from hormone-like increases in luciferase activity but rather represent significant reductions in luciferase expression that likely result from the cytotoxic effects of the dispersants. Statistical significance was determined using analysis of variance followed by t-tests (LSMEANS) using PROC GLM on SAS 9.1 ($p < 0.01$ was used as the critical value to determine statistical significance).

Dispersant	MDA Kb2 cells			CV1 cells		T47D Kbluc cells	
	MTT Cytotoxicity	Androgen Antagonist Assay***	Androgen Agonist Assay	MTT Cytotoxicity	Androgen Agonist Assay	MTT Cytotoxicity	Estrogen Agonist Assay
	LOEC* (ppm)	LOEC (ppm)	LOEC (ppm)	LOEC (ppm)	LOEC (ppm)	LOEC (ppm)	LOEC (ppm)
JD 2000	100	>10	1,000	10,000**	1,000	1,000**	10,000**
DISPERSIT SPC 1000	100	10	100	10	100	10	100
Sea Brat #4	100	>10	100	1,000**	1,000	10,000	1,000
Nokomis 3-AA	100	10	10	1,000	1,000	100	1,000
Nokomis 3-F4	100	>10	10	1,000	1,000	1,000	1,000
Corexit 9500	1,000	>10	1,000	100	1,000	100	1,000
ZI-400	1,000	>10	100	1,000	1,000	1,000	1,000
SAF-RON GOLD	10,000	>10	1,000	10,000	1,000	1,000	10,000

*LOEC (ppm) represents the lowest concentration at which the dispersant consistently reduced the MTT value. Statistical significance was using $p<0.01$ as determined using LSMEANS option of PROC GLM available on SAS 9.1.
** LOEC concentration was equivocal (nonmonotonic response)
*** Antagonist assay for antiandrogens was not run the three highest concentrations (10,000, 1000 and 100 ppm) to avoid most the confounding effects of cell death. The highest concentration was 10 ppm, Dispersants that did not reduce luciferase expression in this assay at any concentration were scored as >10 ppm.

Table 3: Summary table of the Lowest Observed Effect Concentration (LOEC) of the eight dispersants in the MTT cytotoxicity in three cell lines, the estrogen agonist assay in T47D Kbluc cells, the agonist assays in CV-1 and MDA K2 cells and the antagonist assay in MDA Kb2 cells. Since none of the dispersants displayed any effect interpreted as result of the dispersant displaying

endocrine activity we interpret all the results as indications of disruption of cell function and cell death. Since the androgen antagonist assay for antiandrogens did not include the three dispersant highest concentrations (10,000, 1000 and 100 ppm) to avoid most the confounding effects of overt toxicity (seen in the MTT assay with MDA Kb2 cells), the highest concentration in this assay was 10 ppm, Dispersants that did not reduce luciferase expression in this assay at any concentration were scored as >10 ppm. In spite of this precaution, the two most cytotoxic dispersants still reduced luciferase expression in this assay, an effect we attribute to less overt cell toxicity.

The EC_{50} values for the dispersant dose response curves were determined using nonlinear regression procedures with GraphPad Prism 5.0 software (**Figure 5 a,b,c)**. Ranking the eight dispersants in order of highest to lowest potency in the MTT assays and the CPE assessment in three cells lines indicates that there are some consistent differences among dispersants in their ability to disrupt the function and viability of these cell lines (**Figure 5 d**). Dispersant SPC 1000 appears to be more toxic in both MTT (below) and CPE assessments.

Cytotoxicity Summary

For comparison to all of the *in vitro* cytotoxicity assays, we also include LC50 values from whole animal, aquatic species lethality assays for the mysid, *Americamysis bahia*, in a 48-hr static acute toxicity test and an inland silverside, *Menidia beryllina*, 96-hr static acute toxicity test[12]**.** All LC50 values are plotted in **Figure 13** and the numerical values are listed in **Appendix D**. One can see that the cell-based LC50 values overall vary by about two orders of magnitude, and that the values for any given chemical span about one order of magnitude. The rank order of cytotoxicity varied between the various cell types, a not unexpected finding [13]. There is overlap in the range of cytotoxicity for all of the dispersants.

In order to assess, statistically, differential cytotoxicity across the eight dispersants we performed an ANOVA to determine pairwise if any two dispersants were more cytotoxic than the other. We performed this statistical test with and without multiple test correction (Bonferroni). For any dispersant and assay combination that did not achieve an LC50, a default value of 3000 ppm was used; three-fold higher than the highest concentration tested in the relevant assays. LC50 values

greater than 3000 ppm were also set to this default value to prevent large extrapolated LC50 values from biasing the results. All six cell-based quantitative cytotoxicity assays were used for this analysis. The resulting p-values, raw and corrected, are provided in **Table 4**. Both JD 2000 and SAF-RON GOLD tend to be less cytotoxic than the other dispersants. Likewise, DISPERSIT SPC 1000 tends to be more cytotoxic than the other dispersants in the cell-based assays.

The aquatic species LC50 values are almost always lower than the cell-based LC50 values. As with the cell-based assays, JD 2000 is the least toxic in the whole animal assay.

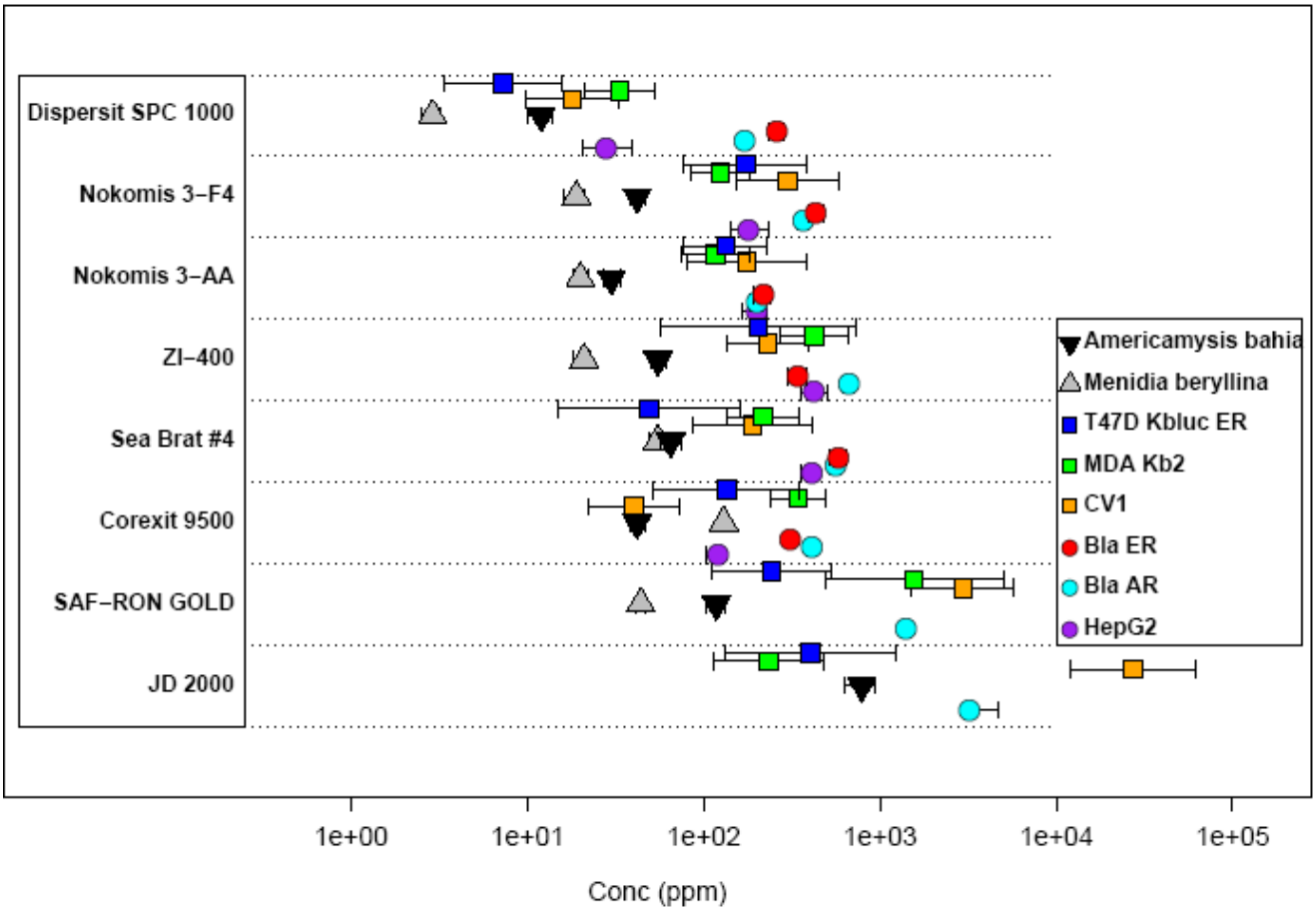


Figure 13: Toxicity data for the dispersants, combining data from cell-based assays in this report with data on aquatic species from a concurrent EPA report [12]. Each horizontal band shows the data for one dispersant. Results are presented from all 6 quantitative cytotoxicity assays. Cell-based LC50 values (concentration at which 50% lethality or effect is observed) are indicated by circles and

squares. Aquatic species LC50 values are indicated by triangles. Note that all dispersants were tested in all assays, and missing data points indicate that no toxicity was seen in that assay at the highest concentration tested. 95% confidence intervals are shown for all assays.

	JD 2000	Dispersit SPC 1000	Sea Brat 4	Nokomis 3-AA	Nokomis 3-F4	Corexit 9500	ZI-400	SAF-RON GOLD
JD 2000		0.1456	0.308	0.1876	0.2464	0.224	0.364	1
Dispersit SPC 1000	0.0052		0.84	1	0.644	1	0.126	0.056
Sea Brat 4	0.011	0.03		1	1	1	1	0.1456
Nokomis 3-AA	0.0067	0.082	0.11		1	1	0.42	0.0756
Nokomis 3-F4	0.0088	0.023	0.5	0.12		1	1	0.1036
Corexit 9500	0.008	0.086	0.34	0.42	0.65		1	0.0952
ZI-400	0.013	0.0045	0.68	0.015	0.19	0.12		0.1652
SAF-RON GOLD	0.92	0.002	0.0052	0.0027	0.0037	0.0034	0.0059	

Raw P-Value

Bonferroni Corrected P-Value

Table 4: 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 pink. All values below the diagonal are raw p-values derived from the ANOVA, while all values above the diagonal swere adjusted for multiple testing.

Other Molecular Targets

In addition to ER and AR, we also analyzed the chemical collection (dispersants plus reference chemicals) using a multiplexed reporter gene assay battery that evaluates activity against a panel of transcription factors including nuclear receptors[5, 9]. These assays were run by Attagene Inc. These data also provide a measure of quality control related to the specificity of any endocrine-related activity caused by the dispersants. The description of the assay and a complete list of targets is given in **Appendix B.2**. All of these assays were carried out twice, one week apart, and in each week, duplicate runs were performed. **Figure 14** summarize all of the results for the dispersants. This plot helps illustrate several key points about the data.

First, as the concentration of a chemical approaches the cytotoxic level, generalized cell stress occurs, accompanied by broad misregulation of transcription. When this threshold is reached, many assays in this system simultaneously activate, but this activity is assumed to be non-specific. One sentinel of this cell stress behavior is NRF2, which is an indicator of generalized oxidative stress. Therefore, if we see many assays become active at about the same concentration, especially if NRF2 is among them, we can discount any target specificity above that concentration. We see this behavior for Corexit 9500 (~50 ppm), JD 2000 (~500 ppm), Nokomis 3-AA (~75 ppm), Nokomis 3-F4 (~75 ppm), Sea Brat #4 (~90 ppm) and ZI-400 (~50 ppm).

The ER activity for Nokomis 3-F4 occurs at a concentration well below where this non-specific behavior is indicated. For ZI-400, the confidence intervals for ER and NRF2 overlap, indicating a possibility that the ER result is non-specific.

The lowest activity that is generally seen is for PXR (Pregnane-X-receptor), which is a xenosensor. This behavior is entirely expected, is common across many classes of organic chemicals, and is not in itself an indicator of toxicity. PXR has been reported to be a xenosensor that acts to protect against endocrine active chemicals[14]. PPAR (peroxisome proliferator activating receptor[15-19]) activity is observed for a number of the dispersants, at higher concentrations than is seen for the PXR assays. There is an extensive literature on PPAR activity

associated with disease in rodents, although the human relevance is unclear [15-18, 20-23]. However, only for Corexit 9500 and Nokomis 3-AA (and potentially for SAF-RON GOLD) is the PPAR signal well below the level of non-specific activity. Vitamin D receptor (VDR) activity is seen for Sea Brat #4 and Nokomis 3-AA below but near the concentration of non-specific behavior.

The activity of JD 2000 cannot necessarily be dismissed as being all non-specific, despite it occurring at the same concentration as NRF2 activity. This is because there are only two target families being activated – PXR and PPAR. A similar observation can be made about DISPERSIT SPC 1000. At the concentration of NRF2 activity, we only see activation of two PXR assays and one for SREBP (SREBF1 sterol regulatory element binding transcription factor 1) which is involved in fatty acid synthesis regulation.

The largest effect (in terms of EMax) of any dispersant and assays is for ZI-400 and AhR (Aryl hydrocarbon receptor), with EMax >30. The AhR is well-known for its role in mediating the adaptive metabolism of xenobiotics, and also in the toxicity that follows exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin). This indicates the potential for the presence of a dioxin-like compound, which would be cause for concern. In the ToxCast Phase I data set[4, 5] of 309 chemicals, we saw only three with AhR efficacy higher than is seen with ZI-400. It is not clear though that this effect is specific, given that it occurs in the same concentration range as activity in a number of other targets, and above the NRF2 AC50.

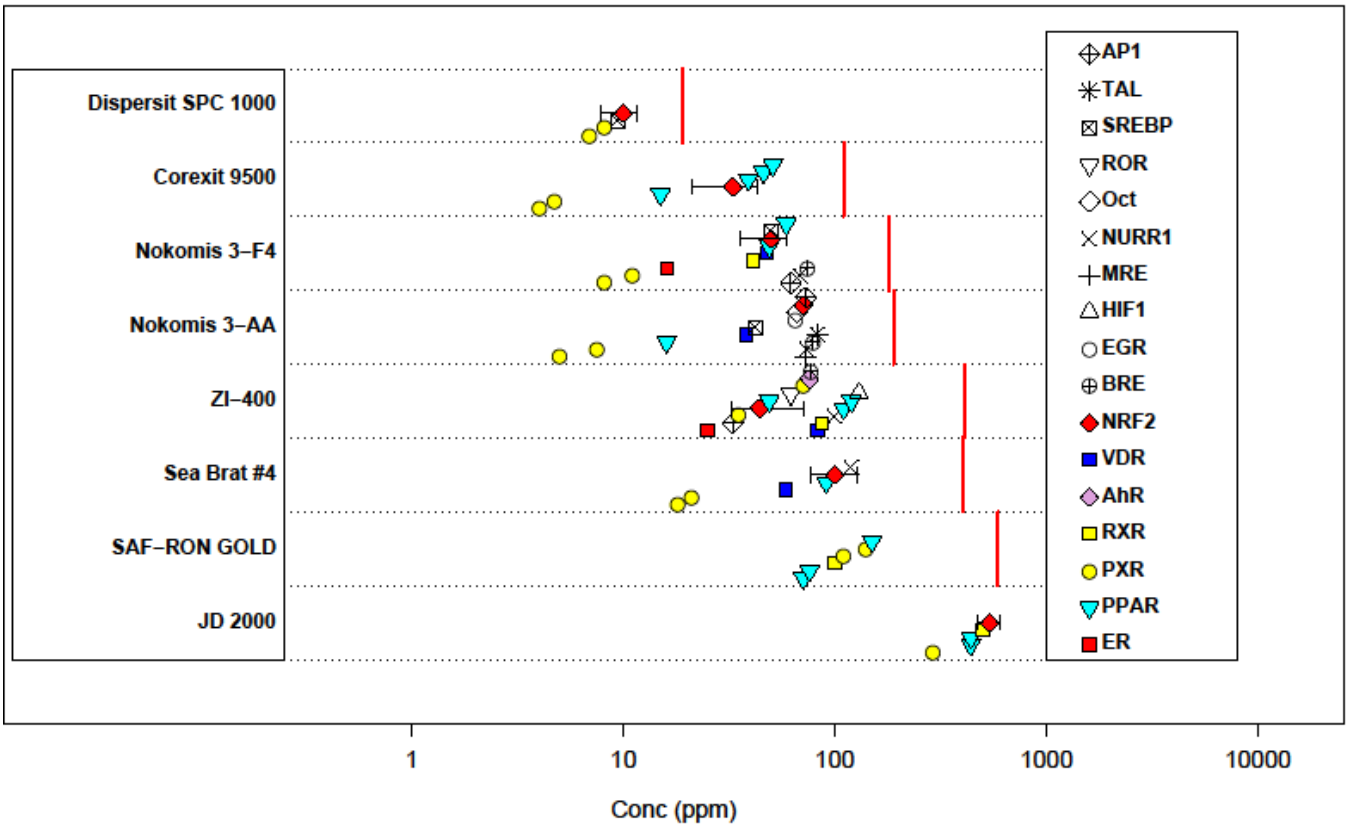


Figure 14: Summary plot of all Attagene *cis* and *trans* assays for dispersants with AC50 values below cytotoxicity levels. Each horizontal band displays data for a single dispersant. The x-value is the AC50. Points are staggered in the y-direction to make overlapping points visible. Where there were multiple assays for a given gene target (e.g. PPARα, PPARδ, PPARγ) were given a single symbol. For illustration, 95% confidence intervals are shown on assays for NRF2. The vertical red lines indicate the LC50 for cytotoxicity in the HepG2 cells**.** Dispersants are ordered by decreasing cytotoxicity LC50 values.

The major conclusions of this section are that several of the dispersants display PXR and PPAR activity at concentrations below where cell stress and cytotoxicity occur. These are expected responses in hepatocytes to xenobiotics. The ordering of dispersants by lowest concentration at which bioactivity occurs is consistent with the ordering based on cytotoxicity. One observation of more general interest is that we are able to detect specific target-based bioactivity in complex mixtures such as these. This is observation is relevant to the challenges of real world chemical toxicity testing, wherein humans and other organisms are often exposed to

complex mixtures rather than the pure single compounds that are the subject of typical toxicity testing.

Conclusions

The primary conclusions are as follows:

For six of the eight dispersants tested we found no evidence that they would be capable of interacting with estrogen or androgen receptor function from testing in multiple *in vitro* systems. For the other two dispersants, there was a weak ER signal in one assay. However, integrating over all of the ER and AR results, these data do not indicate that any of the eight dispersants will display biologically significant endocrine activity via the androgen or estrogen signaling pathways. As mentioned previously, NPEs (and their breakdown product NPs) can be endocrine disruptors in fish[1], so the risk of using NPE-containing dispersants should be carefully weighed against the expected benefits. One limitation of the present study is that there are other routes by which chemicals can cause endocrine disruption, as well as other types of toxicity that have not been tested for here. Most importantly though, there were no indications of estrogenic activity for Corexit 9500, the dispersant currently being used in the Gulf of Mexico.

All of the dispersants showed cytotoxicity in at least one cell type at concentrations between 10 and 1000 ppm. Both JD 2000 and SAF-RON GOLD tend to be less cytotoxic than the other dispersants. Likewise, DISPERSIT SPC 1000 tends to be more cytotoxic than the other dispersants in the cell-based assays. The aquatic species LC50 values tend to be lower than the cell-based LC50 values. As with the cell-based assays, JD 2000 is the least toxic in the whole animal assay.

Supplementary Information

Supplemental information, including a QA Statement, is included in the referenced Appendices.

QA Summary

All research described in this report was conducted under a comprehensive and rigorous program of quality assurance (QA), as documented in the QA supplemental file. The overall goal of the QA program was to ensure research data were of known and acceptable quality. QA staff surveillance of critical research activities was an important feature of the overall QA approach and ensured quick and effective resolution of any problems. The conclusion of the QA review process is that results presented in this report accurately reflect the raw data obtained during the course of the research and are scientifically valid and defensible.

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