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Understanding the implications of dissolved organic carbon when assessing antagonism *in vitro*: An example with an estrogen receptor assay

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Abstract

Both estrogenic and anti-estrogenic activity has been observed in water samples. Some studies have suggested that dissolved organic carbon (DOC), which can be co-extracted during sample enrichment, contributes to the apparent antagonistic effect. DOC has a high sorption capacity for the estrogen receptor (ER) agonist 17β-estradiol, which may reduce the available 17β-estradiol concentration in the antagonist testing mode and potentially lead to apparent antagonism. The aim of the study was to determine the influence of DOC when assessing antagonism in an ER reporter gene assay. The presence of DOC shifted the 17β-estradiol concentration-effect curve to higher concentrations, increasing the nominal EC_{50} value by up to 0.3 log units. However, this shift was within the usual variability associated with repeated measurements of concentration-effect curves. This shift was not due to DOC being an antagonist itself or interfering with fluorescence measurements, but was due to DOC reducing the bioavailability of 17β-estradiol. This was demonstrated by modelling the DOC sorption corrected 17β-estradiol concentration using experimental DOC-water partition coefficients (K_{DOC}). While the shift in the 17β-estradiol concentration-effect curve was minor, sorption of 17β-estradiol to DOC can have an impact when assessing antagonism. At the EC_{50} agonist concentration, both modelled and experimental results showed that DOC at concentrations similar to that co-extracted in water samples caused suppression of the agonist at levels that would be classified as antagonism. The suppression was less pronounced at the EC_{80} agonist concentration, hence this is recommended when assessing antagonism of DOC rich samples, such as surface water and wastewater.

Keywords: antagonism; dissolved organic carbon; estrogen receptor; *in vitro*

1. Introduction

The aquatic environment contains countless micropollutants from sources including wastewater effluent and agricultural run-off (Schwarzenbach et al., 2006). Of particular concern for aquatic wildlife and human health are endocrine disrupting chemicals, which include natural and synthetic hormones, as well as some industrial compounds and pesticides (Bergman et al., 2013). *In vitro* reporter gene assays, such as the yeast estrogen screen (YES) and ER-CALUX, are commonly applied to assess both estrogenic and anti-estrogenic activity of water samples, including wastewater and surface water (van der Linden et al., 2008; Zhao et al., 2011; Scott et al., 2014). In recent years, many studies have observed both estrogenic and anti-estrogenic effects in water samples (e.g. Ihara et al., 2014; Rao et al., 2014) and this has been attributed to a range of factors from the presence of industrial compounds (e.g. Fang et al., 2012) to potential matrix effects from organic matter (e.g. Conroy et al., 2007).

Surface water and wastewater can contain high levels of dissolved organic carbon (DOC) and this can be co-extracted with other organic contaminants during solid phase extraction (SPE), which is often used for sample enrichment prior to bioanalysis. Studies focusing on reference DOC, which should not contain micropollutants, have also observed apparent anti-estrogenic (Janosek et al., 2007; Wu et al., 2009) and anti-androgenic effects (Bittner et al., 2012). There are several possibilities to explain these observations: either 1) DOC can act as an antagonist, or DOC interferes with an assay parameter causing experimental artefacts, for example 2) the properties of DOC, such as autofluorescence, interfere with the reporter gene assay measurement, or 3) DOC modulates the agonist concentration used in the assay.

The potential for DOC to alter the agonist concentration (option 3) has not been evaluated in the literature to date, despite it being plausible, given the experimental methodology applied to assess antagonism for reporter gene assays. A constant concentration of agonist, such as 17β-estradiol for the estrogen receptor (ER) assay, is added and any antagonistic compounds in the sample can compete with the agonist for binding sites, leading to inhibition of the agonist background. The agonist concentration used in bioassays run in antagonist mode can vary from the concentration causing 50% effect (EC_{50}) up to EC_{100} (van der Linden et al., 2008; Ihara et al., 2014). The commonly used ER agonist 17β-estradiol, which is a moderately hydrophobic compound, can sorb to DOC, with DOC-water partition coefficients (K_{DOC}) ranging from 5.1×10^3 to 1.9×10^5 L/kg, depending on the DOC properties (Yamamoto et al., 2003; Neale et al., 2008). Given the strong sorption capacity of DOC, it is possible that the reported anti-estrogenic effects in natural waters are related to the presence of co-extracted DOC, which can decrease the available 17β-estradiol

concentration and cause the apparent antagonism. Buckley (2010) attempted to exclude the influence of co-extracted DOC by filtering wastewater SPE extracts through a membrane with a 1000 Da molecular weight cut-off. While some fractions of DOC are larger than 1000 Da, such as biopolymers, many fractions are smaller, including humic substances and low molecular weight neutrals (Huber et al., 2011), thus such a filtration processes is unlikely to remove a significant fraction of the total DOC.

With a specific focus on the ER assay, this study aimed to test the hypothesis that the reported antagonism in the presence of reference DOC is caused by the reduction of the unbound agonist concentration due to sorption to DOC. This was explored using both an experimental and modelled approach by applying experimental K_{DOC} values. Suwannee River humic acid (HA) and fulvic acid (FA) were selected as representative DOC. The study also investigated the implications of co-extracted DOC when assessing antagonism *in vitro* for DOC rich samples.

2. Materials and Methods

2.1. Chemicals

All chemicals were of analytical grade and were purchased from Sigma Aldrich (Castle Hill, Australia), unless otherwise specified. Suwannee River HA (2S101H) and FA (2S101F) standards from the International Humic Substance Society (St. Paul, US) were used as reference DOC.

2.2. Predicting 17β-estradiol binding to dissolved organic carbon

The amount of 17β-estradiol binding to DOC was estimated using experimental DOC-water partition coefficients (K_{DOC}) from Neale et al. (2008). K_{DOC} is defined as the ratio of the 17β-estradiol concentration sorbed to DOC ($C_{DOC,}$ ng/kg) to the aqueous 17β-estradiol concentration (C_W, ng/L) (Equation 1), where n_{DOC} is the amount sorbed to DOC (ng), n_W is the amount in water (ng), m_{DOC} is the mass of DOC in the system (kg) and V_w is the volume of water (L).

$$K_{DOC} = \frac{C_{DOC}}{C_W} = \frac{n_{DOC}}{m_{DOC}} \cdot \frac{V_W}{n_W} \quad (1)$$

The applied log K_{DOC} values were 4.04 and 3.78 L/kg for HA and FA, respectively. The fraction of 17β-estradiol sorbed to DOC (f_{DOC}) can be calculated using Equation 2 (Neale et al., 2011).

$$f_{DOC} = \frac{n_{DOC}}{n_{DOC} + n_W} = \frac{1}{1 + \frac{V_w}{(m_{DOC} + K_{DOC})}}$$

(2)

This equation can be re-arranged to calculate the mass of DOC required to bind a certain fraction of 17β-estradiol (Equation 3).

$$m_{DOC} = \frac{V_w}{\frac{1}{(f_{DOC} - 1)}} \cdot \frac{1}{K_{DOC}}$$

(3)

The mass of DOC predicted to sorb 20 to 60% of 17β-estradiol ranged from 9.12×10^{-10} to 5.47×10^{-9} kg for HA and 1.66×10^{-9} to 9.96×10^{-9} kg for FA. This translates to 22.8 to 136.8 mg_C/L and 41.5 to 248.9 mg_C/L for HA and FA, respectively, based on a final volume of 40 µL in the assay.

The concentration of 17β-estradiol ($C_{DOC\text{-}sorption\ corrected}$ (E2)) in the presence of DOC in the assay was calculated from the nominal (i.e., added) concentration ($C_{nominal}$ (E2)) and f_{DOC} (Equation 4). It is important to note that $C_{DOC\text{-}sorption\ corrected}$ (E2) relates only to sorption to DOC and binding to media components, such as cells and serum, was neglected in the current study.

$$C_{DOC\text{-}sorption\ corrected}(E2) = (1 - f_{DOC}) \cdot C_{nominal}(E2)$$

(4)

2.3. ER assay

The GeneBLAzer® ERα-UAS-bla assay (Life Technologies, Mulgrave, Australia) was used in the current study. The HEK 293T cells were grown in DMEM with GlutaMAX™ supplemented with 10% dialysed fetal bovine serum (FBS), while the assay media was phenol red-free DMEM with 2% charcoal stripped FBS. Ten point concentration-effect curves of agonist 17β-estradiol were prepared across a 96 well plate using a 2.5×10^{-5} M 17β-estradiol methanol stock, with media only in the last two columns (plate A), while a second 96 well plate contained HA or FA dissolved in phenol red-free DMEM at concentrations expected to cause 20 to 60% 17β-estradiol binding in the final 384 well plate (plate B). Due to subsequent dilution steps, plates A and B were prepared at concentrations 10 times higher than the final assay concentration. Fifty microliters from each plate

A and B were mixed together in a separate 96 well plate and 8 µL was added in duplicate to a 384 well black clear bottom plate containing 32 µL of cells at a density of 6.2×10^5 cells/mL. The solvent concentration in the 384 well plate was 0.2%. For validation, each plate contained agonist 17β-estradiol and antagonist tamoxifen (with EC_{80} 17β-estradiol constant background) concentration-effect curves, along with solvent controls and cell free controls. The cells were incubated overnight in a 5% CO_2 incubator at 37 °C. The following day 8 µL of LiveBLAzer™ FRET substrate mixture was added to each well. Fluorescence at 460 and 520 nm was measured after excitation at 409 nm using a Fluostar Omega (BMG Labtech, Ortenberg, Germany) at time zero and after 2 h. The fluorescence of the cell-free controls was subtracted from the samples for each wavelength and the emission ratio of blue (460 nm) to green (520 nm) (B/G) was calculated (Equation 5), where F is fluorescence.

$$B/G = \frac{F_{blue}(T=2) - F_{blue,cell\text{-}free}(T=2)}{F_{green}(T=2) - F_{green,\ cell\text{-}free}(T=2)} \quad (5)$$

To assess if the properties of HA and FA were interfering with the fluorescence measurements, the autofluorescence corrected B/G emission ratio ($B/G_{autofluorescence\text{-}corrected}$) was calculated using Equation 6. The addition of the FRET reagent changed the emission spectra of HA and FA. Therefore, fluorescence of the samples measured immediately after the addition of LiveBLAzer™ substrate, minus the fluorescence of the unexposed control, was subtracted from fluorescence of the samples measured after 2 h for both the blue and green output to correct for possible interference by autofluorescence of the sample.

$$B/G_{autofluorescence\text{-}corrected} = \frac{F_{blue}(T=2) - (F_{blue}(T=0) - F_{blue\text{-}unexposed\ control}(T=0))}{F_{green}(T=2) - (F_{green}(T=0) - F_{green\text{-}unexposed\ control}(T=0))} \quad (6)$$

2.4. Concentration-effect modelling

The B/G emission ratio could be converted to percent effect related to the maximum response (% effect) using Equation 7 with the B/G emission ratio of the unexposed control ($B/G_{unexposed\ control}$) and maximum B/G emission ratio ($B/G_{maximum}$).

$$\%\ \text{Effect} = \frac{(B/G - B/G_{\text{unexposed control}})}{(B/G_{\text{maximum}} - B/G_{\text{unexposed control}})} \quad (7)$$

The concentration-effect curve was modelled using Equation 8, using parameters from an average 17β-estradiol concentration-effect curve, including EC_{50} and slope, and C (E2), which can either be C_{nominal} (E2) or $C_{\text{DOC-sorption corrected}}$ (E2) (Equation 4). The concentration-effect curve was modelled for DOC concentrations expected to cause 20 to 60% binding.

$$\%\ \text{Effect} = \frac{1}{1+10^{(\text{slope}\cdot(\log EC_{50} - \log C(E2)))}} \quad (8)$$

3. Results and Discussion

3.1. Influence of DOC on ER assay

When assessed in classical agonist mode, neither HA nor FA alone caused an effect in the ER assay, indicating that they are not estrogenic. This was not unexpected and has been observed previously by Janosek et al. (2007) and Chen et al. (2012). However, when either HA or FA was added to nominal 17β-estradiol concentration-effect curves at concentrations expected to cause 20 to 60% binding to 17β-estradiol, the curves shifted to the right, leading to higher nominal 17β-estradiol EC_{50} values in the presence of DOC (Figure 1 (HA only) and Figure SI-1 (FA and replicate HA experiment)).

The amount of HA and FA added ranged from 22.8 to 136.8 mg_C/L and 41.5 to 248.9 mg_C/L, respectively. Tanghe et al. (1999) also observed an increase in 17β-estradiol and nonylphenol EC_{50} values in the presence of 150 mg_C/L of HA. Similarly, Holbrook et al. (2005) observed an increased 17β-estradiol EC_{50} value in the presence of colloidal organic carbon from a wastewater treatment plant and attributed this to the reduction in 17β-estradiol bioavailability due to sorption. In the current study, the presence of DOC increased the nominal 17β-estradiol EC_{50} value by up to 0.3 log units. However, it should be noted that the difference in EC_{50} values in the presence of DOC was within the usual variability observed for 17β-estradiol concentration-effect curves between different assay runs.

3.2. Does DOC autofluorescence interfere with assay measurement?

The spectral properties of DOC are known to interfere with bioassay measurements. For example, HA and FA autofluorescence can interfere with chlorophyll fluorescence measurements using imaging pulse amplitude modulation (PAM) fluorometry (Neale and Escher, 2014). Further, Oosterom et al. (2005) also found that coloured compounds can interfere with FRET measurements by increasing green fluorescence only, leading to a reduced B/G emission ratio. The potential for HA and FA to interfere with the fluorescence measurement was assessed by subtracting the background fluorescence at T=0. There was very little difference in the concentration-effect curves with and without autofluorescence correction (Figure 2 (50% HA only) and Figure SI-2 (remainder of HA and FA)), and the difference between corresponding EC_{50} values was less than 0.05 log units. This indicates that the observed shift in the concentration-effect curve was not due to DOC autofluorescence causing an experimental artefact. Given that DOC does not appear to interfere with the fluorescence measurements, the results will be expressed as percent effect related to the maximum response from here on.

3.3. Is DOC a non-competitive antagonist?

The fact that neither HA nor FA significantly shifted the 17β-estradiol concentration-effect curve to the right indicates that they are not competitive antagonists. However, the presence of both HA and FA, irrespective of concentration, suppressed the maximal B/G emission ratio response. This occurrence has been previously attributed to the presence of non-competitive antagonists (Ihara et al., 2014).

However, the observed decrease in maximal response did not occur in a dose-dependent manner, which suggests that DOC is not a non-competitive antagonist. Instead, it is possible that HA and FA is inferring with the hydrolysis of fluorescent FRET substrate. ER agonist exposed cells produce enzyme beta-lactamase, which cleaves the fluorescent substrate and increases the blue fluorescence signal. Previous studies have shown that the presence of HA and FA can reduce enzymatic cleavage of proteolytic enzyme Pronase E (Jahnel and Frimmel, 1994).

To test this hypothesis, cells were exposed to 17β-estradiol without DOC overnight in a 384 well plate, with HA at concentrations expected to cause 20 to 60% binding added only with the FRET substrate. Fluorescence was measured after 2 h exposure at room temperature. The addition of HA in the FRET substrate did not cause the concentration-effect curve to shift to the right, but the maximal response, now expressed as percent effect, was lower in the presence of HA (Figure 3).

This supports the hypothesis that DOC can interfere with the cleavage of the fluorescent substrate at high beta-lactamase concentrations.

3.4. Contribution of sorption to DOC

The observed shift the 17β-estradiol concentration-effect curve in the presence of DOC does not appear to be related to non-competitive antagonism or the spectral properties of DOC. To test if the increased nominal EC_{50} values observed in the presence of DOC were due to reduced 17β-estradiol bioavailability, $C_{DOC\text{-}sorption\ corrected}$ (E2) was predicted based on experimental K_{DOC} values taken from Neale et al. (2008). The K_{DOC} values were quantified at environmentally relevant DOC concentrations using negligible depletion solid-phase microextraction (nd-SPME) (Neale et al., 2008). The experimental and modelled concentration-effect curves were plotted using both $C_{nominal}$ (E2) and $C_{DOC\text{-}sorption\ corrected}$ (E2) (Figure 4). For this comparison, the slope of the concentration-effect curves was fixed to that of 17β-estradiol in DOC-free media (0.82 and 0.88 in replicate experiments, respectively). When plotted using $C_{DOC\text{-}sorption\ corrected}$ (E2), the experimental and modelled concentration-effect curves overlapped and there was no observed shift to the right. This suggests that the observed increase in nominal EC_{50} values in the presence of DOC was related to 17β-estradiol binding to DOC and consequently reducing its bioavailability.

3.5. Implications of co-extracted DOC when assessing antagonism

As demonstrated above, the presence of DOC can reduce the available concentration of agonist 17β-estradiol, but the implications of this when assessing antagonism in *in vitro* assays, where a nominal agonist concentration is added, is unclear. The nominal agonist concentration can vary significantly between studies and can range from EC_{50} to EC_{100}. To assess the influence of agonist sorption to DOC, the change in percent effect of the nominal EC_{50} and EC_{80} agonist concentrations in the presence of HA was shown in Figure 5A (data for FA in Figure SI-4A). Both the modelled and experimental results showed a decrease in agonist response, with a greater decrease with increasing DOC concentration, though there was considerable variability associated with the experimental results.

When assessing antagonism, a cut-off of 20% suppression of the background agonist concentration is often applied, with samples suppressing the agonist concentration by more than 20% considered to be antagonists. To determine whether 17β-estradiol sorption to DOC could lead to apparent antagonism, percent effect was converted to percent suppression for both the nominal EC_{50} and EC_{80} agonist concentrations (Figure 5B (HA only) and Figure SI-4B (FA)). Both the modelled and experimental results showed that suppression was more pronounced for the EC_{50} agonist

concentration, with the HA concentrations binding up to 50 to 60% of 17β-estradiol leading to as much as 35% suppression. This would most likely be reported as detectable antagonism. This effect was less pronounced at the EC_{80} agonist concentration, with around 20% suppression at the highest HA concentration. The difference between EC_{50} and EC_{80} is because the rate of change as a function of concentration is greatest at EC_{50}, hence small changes will have a bigger influence at EC_{50} compared to EC_{80}, as recently illustrated by Neale and Leusch (2015).

To put the results into context for environmental water samples, the amount of co-extracted DOC typically added to the ER assay was estimated. This can depend on a number of factors including DOC concentration in the source water, DOC extraction efficiency by SPE and dilution in assay. The DOC concentration in surface water and wastewater can vary considerably and can range from 1 to 100 mg_C/L (Steinberg et al., 2006). For illustrative purposes, a DOC concentration of 10 mg_C/L was assumed and in this example 1 L of water was enriched to a final volume of 0.5 mL using SPE to give an enrichment factor of 2000. Regarding SPE extraction efficiency, this can be influenced by the SPE material and DOC properties. Previous work on co-extraction of wastewater derived DOC by Oasis HLB cartridges, a commonly used SPE material for complex water samples, showed DOC extraction efficiency can vary from 40-70% (Neale and Escher, 2014). Consequently, DOC extraction efficiencies of 40 and 70% were applied for this example. Finally, the amount of dilution in the assay can vary depending on sample potency and solvent effects. For this example, 100 times dilution in the assay was assumed, as this is commonly used for environmental samples in the applied assay. Applying these parameters gives a maximum DOC concentration in the assay ranging from 80 to 140 mg_C/L. Based on Figure 5, sorption to DOC in this concentration range could lead to greater than 20% suppression of the agonist concentration, particularly if an EC_{50} agonist concentration is used, and an erroneous description of the sample as "containing anti-estrogenic compounds".

However, it should be noted that DOC from other sources can have different sorption capacities compared to HA and FA. For example, previous work by Neale et al. (2011) found that the sorption capacity of wastewater derived DOC was less than reference HA. Hence, the role of sorption is expected to be less for wastewater derived DOC.

4. Conclusions

In this study, we applied an experimental and modelled approach to demonstrate that sorption to DOC can reduce the available 17β-estradiol concentration and lead to increased nominal EC_{50} values. This was not due to antagonism or experimental artefacts from DOC autofluorescence. The

observed shift in the 17β-estradiol concentration-effect curve in the presence of DOC was within the variability associated with DOC-free concentration-effect curves over time. However, the influence of sorption to DOC was not insignificant when assessing apparent antagonism, with DOC concentrations expected to cause 50 to 60% binding resulting in up to 35% suppression of the EC_{50} agonist concentration. This is relevant for environmental samples as the final DOC concentration in the assay is expected to be in the same concentration range for DOC rich water samples, such as wastewater, and for complex environmental samples only nominal concentrations in units of relative enrichment factors can be reported.

The influence of DOC in antagonist mode was explored for the ER reporter gene assay as it is a commonly used endpoint and experimental K_{DOC} values are available for 17β-estradiol with HA and FA. The observed shift in the agonist concentration-effect curve is also likely for other assays where a moderately hydrophobic compound is used as the agonist (e.g. progestagens such as levonorgestrel in the progesterone receptor assay and androgens such as dihydrotestosterone in the androgen receptor assay) and this may explain the reported anti-androgenicity of organic matter observed by Bittner et al. (2012). However, reliable K_{DOC} values are lacking for these compounds, making it difficult to test this hypothesis.

When assessing antagonism in DOC rich water samples, such as wastewater, using an agonist concentration of EC_{80} rather than EC_{50} is recommended to limit the potential influence of co-extracted DOC. Further, to assess if sorption to DOC could be a factor, studies should measure the DOC concentration of the environmental samples, at least prior to SPE. This is often overlooked, but could be used as a screening step to help researchers identify whether DOC could be potentially interfering with their experiment.

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Figure 1: Nominal 17β-estradiol (E2) concentration-effect curves in the presence of humic acid (HA) concentrations, which are expected to cause 20 to 60% binding, compared to DOC free media.

Figure 2: Nominal 17β-estradiol (E2) concentration-effect curves with humic acid (HA) expected to causing 50% binding with (open symbols) and without (solid symbols) autofluorescence correction.

Figure 3: Nominal 17β-estradiol (E2) concentration-effect curves with humic acid (HA) at concentrations expected to cause 20 to 60% binding only dosed in the FRET reagent after overnight exposure compared with a DOC free control.

Figure 4: Experimental (symbols) and modelled (dashed lines) concentration-effect curves for both nominal ($C_{nominal}$ (E2)) and DOC sorption corrected ($C_{DOC\text{-}sorption\ correction}$ (E2)) concentrations in the presence of (A, C) humic acid (HA) and (B, D) fulvic acid (FA) (typical experiment). Vertical dotted lines indicate the EC_{50} and EC_{80} concentrations for the E2 only concentration-effect curve.

Figure 5: Experimental and modelled (A) percent effect and (B) percent suppression of the nominal agonist concentration at EC_{50} (black, closed symbols) and EC_{80} (blue, open symbols) as a function of humic acid (HA) concentration.

Figure 1

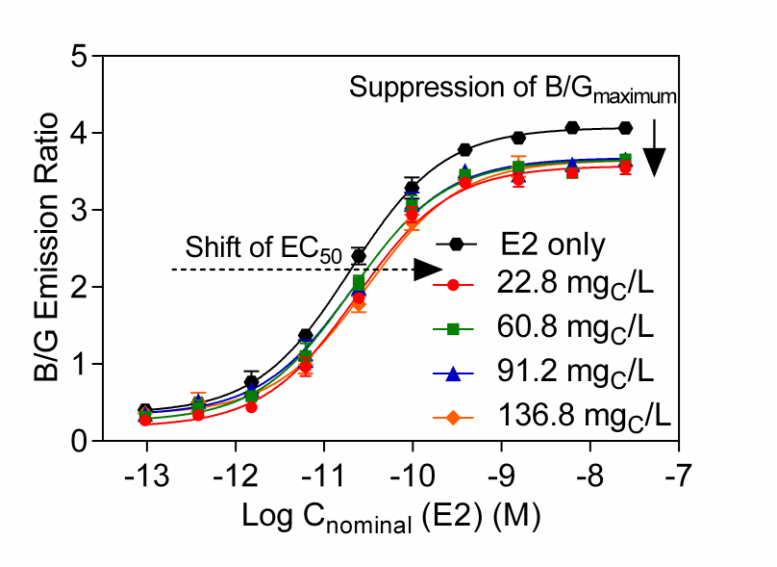

Suppression of B/G$_{maximum}$
Shift of EC$_{50}$
E2 only
22.8 mg$_C$/L
60.8 mg$_C$/L
91.2 mg$_C$/L
136.8 mg$_C$/L
B/G Emission Ratio
Log C$_{nominal}$ (E2) (M)
0
1
2
3
4
5
-13
-12
-11
-10
-9
-8
-7

Figure 2

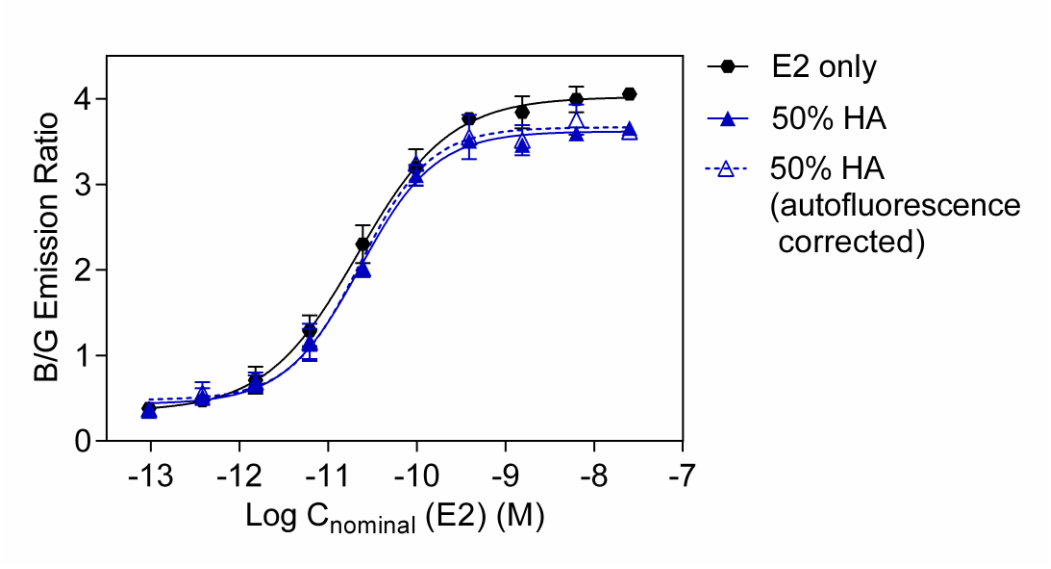

B/G Emission Ratio
0
1
2
3
4
-13
-12
-11
-10
-9
-8
-7
Log C nominal (E2) (M)
E2 only
50% HA
50% HA
(autofluorescence
corrected)

Figure 3

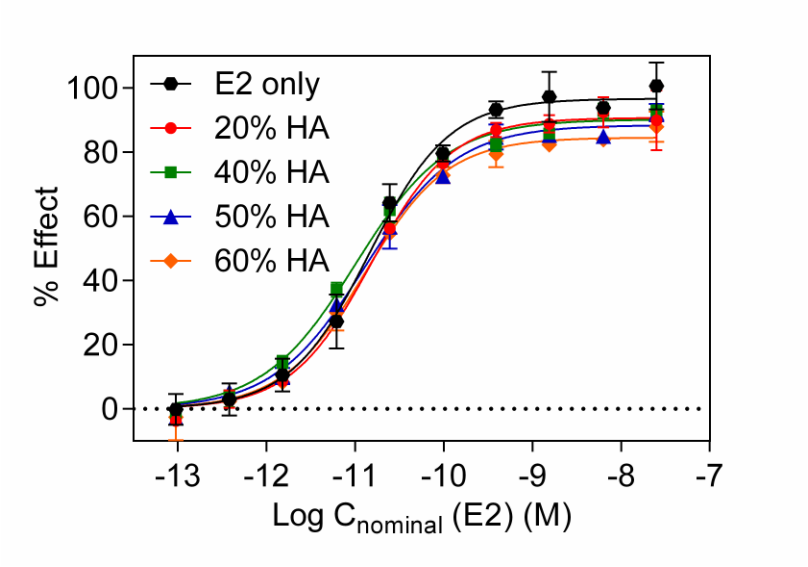
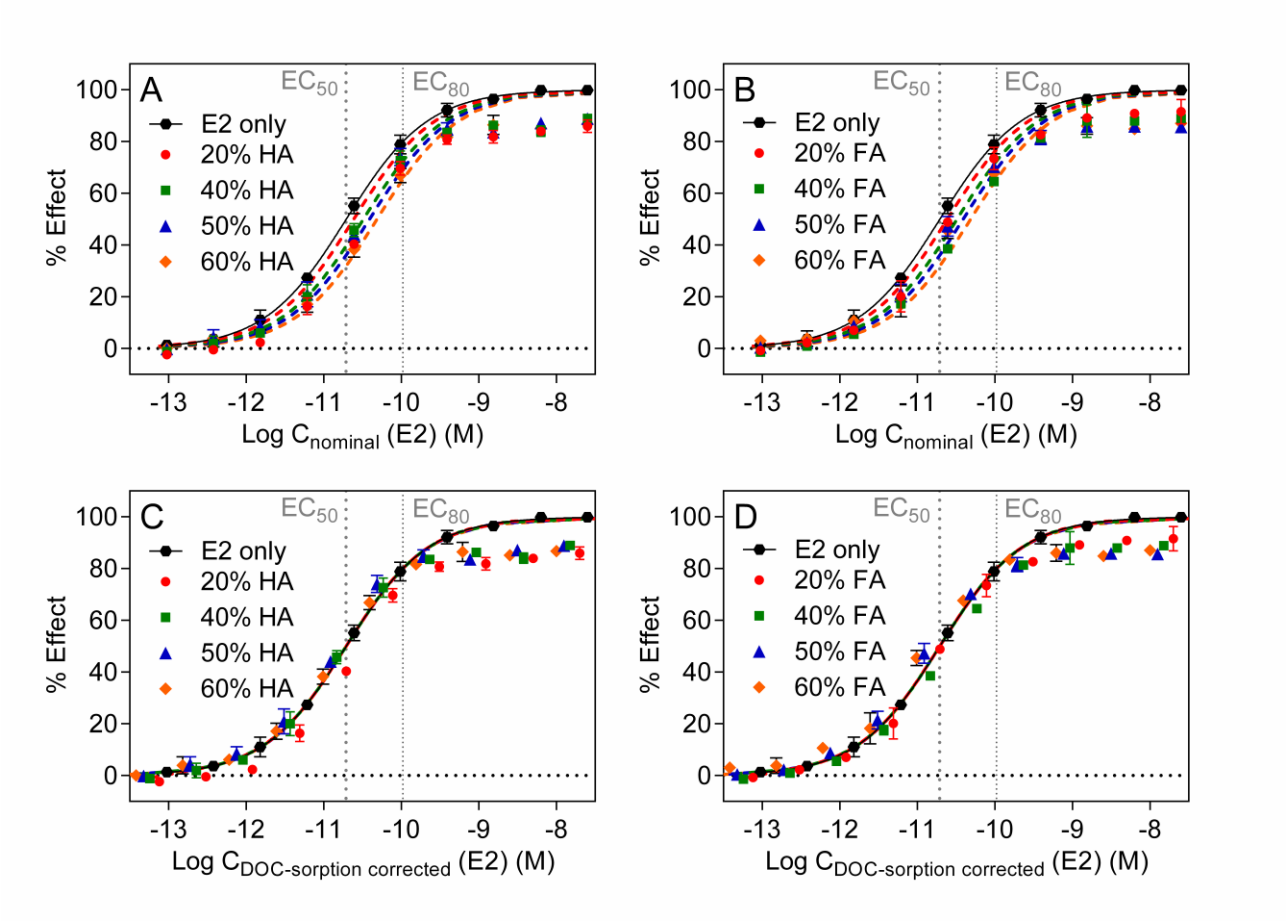

E2 only
20% HA
40% HA
50% HA
60% HA
% Effect
100
80
60
40
20
0
-13
-12
-11
-10
-9
-8
-7
Log C nominal (E2) (M)

Figure 4


A
B
C
D
EC50
EC80
E2 only
20% HA
40% HA
50% HA
60% HA
20% FA
40% FA
50% FA
60% FA
% Effect
Log Cnominal (E2) (M)
Log CDOC-sorption corrected (E2) (M)

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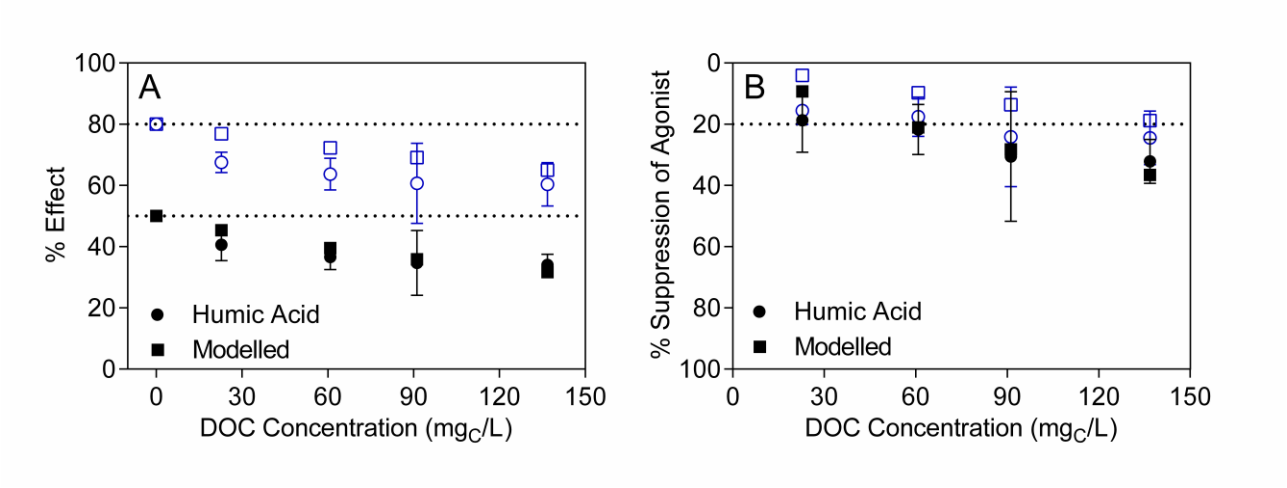

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