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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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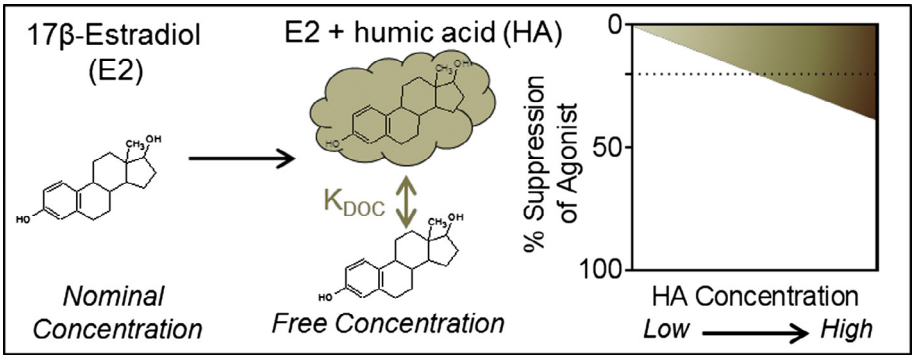
HIGHLIGHTS

- Dissolved organic carbon stated to cause apparent antagonism in *in vitro* bioassays.
- DOC shifted 17β-estradiol (E2) concentration-effect curves to higher concentrations.
- Shift was not due to DOC being an antagonist or interfering with bioassay reading.
- Increase in EC_{50} was due to DOC reducing the bioavailable E2 concentration.
- DOC in water samples may cause E2 suppression and be reported as anti-estrogenic.

GRAPHICAL ABSTRACT



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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

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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.

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)) (Eq. (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 Eq. (2) (Neale et al., 2011).

$$f_{DOC} = \frac{n_{DOC}}{n_{DOC} + n_W} = \frac{1}{1 + \frac{V_w}{(m_{DOC} \cdot K_{DOC})}} \quad (2)$$

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

$$m_{DOC} = \frac{V_w}{\frac{1}{(f_{DOC}-1)}} \cdot \frac{1}{K_{DOC}} \quad (3)$$

The mass of DOC predicted to sorb 20–60% 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 22.8–136.8 mg_C/L and 41.5–248.9 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} (Eq. (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.

$$C_{DOC\text{-}sorption\ corrected}(E2) = (1 - f_{DOC}) \cdot C_{nominal}(E2) \quad (4)$$