Consideration when assessing antagonism *in vitro*: Influence of agonist concentration and dissolved organic carbon

Peta A Neale[1,2], Beate I Escher[2,3,4], Frederic DL Leusch[1]

[1]Smart Water Research Centre, Australian Rivers Institute, School of Environment, Griffith University, Southport Qld 4222, Australia; [2]The University of Queensland, National Research Centre for Environmental Toxicology (Entox), 39 Kessels Rd, Brisbane Qld 4108, Australia; [3]Helmholtz Centre for Environmental Research, UFZ, Leipzig, Germany; [4]Eberhard Karls University Tübingen, Environmental Toxicology, Center for Applied Geosciences, Tübingen, Germany

Introduction

- There is increasing recognition of the importance of assessing antagonism in parallel with agonism *in vitro* for environmental water samples.
- Currently, the assessment of antagonism is limited due to the lack of a standardised approach, with the added agonist concentration ranging from the concentration causing 50% effect (EC_{50}) to the maximal effect (EC_{100}).
- Further, environmental water samples can contain high levels of dissolved organic carbon (DOC), which has been suggested to cause apparent antagonism *in vitro.*
- This may be due to DOC reducing the bioavailable agonist concentration as DOC can sorb moderately hydrophobic agonists, such as 17β-estradiol (E2) in the estrogen receptor (ER) assay.

This study aims to investigate how changing agonist concentrations and the presence of DOC can influence antagonism *in vitro* using the example of an ER reporter gene assay

Methodology

- ER assay: GeneBLAzer® ERα-UAS-bla assay.

Influence of agonist concentration

- Antagonist tamoxifen (TMX) concentration-effect curves were prepared with background E2 concentrations ranging from 6.3×10^{-12} to 2.5×10^{-8} M.

Influence of DOC

- Suwannee River humic acid (HA) was used as reference DOC.
- Experimental DOC-water partition coefficients (K_{DOC}) from Neale et al. (2008, ES&T, 42: 2886) were used to predict E2 binding to HA.
- The E2 concentration in the presence of DOC ($C_{DOC\text{-}sorption\ corrected}$ (E2)) was predicted using K_{DOC} and the nominal E2 concentration ($C_{nominal}$ (E2)).
- E2 concentration-effect curves were prepared with HA expected to cause 20 to 60% binding of E2.
- E2 concentration-effect curves were modelled in the presence of DOC using $C_{DOC\text{-}sorption\ corrected}$ (E2) or $C_{nominal}$ (E2) and parameters from an average E2 concentration-effect curve, including E2 EC_{50} and slope.

Influence of changing agonist concentration

- Different competing E2 concentrations resulted in a shift in antagonistic effect (Fig 1).
- The TMX EC_{50} value varied from log -6.4 to -4.9 M with added E2 concentrations from EC_{50} to EC_{100}.
- While lower agonist concentrations (e.g. EC_{50}) increase the sensitivity of the assay, the 50% effect level is the area of the concentration-effect curve most influenced by minimal changes, as indicated by the ratio of change of the response as a function of concentration (Fig 2).
- Consequently, small errors in dosing can have a greater influence on assay variability at EC_{50}.
- As assay sensitivity is similar at both the EC_{50} and EC_{80} agonist concentrations and EC_{80} is less susceptible to variability, the EC_{80} agonist concentration is recommended when assessing antagonism *in vitro.*

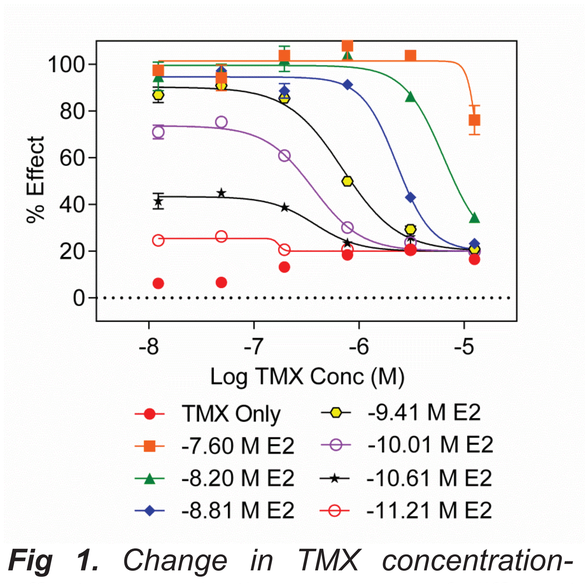

Fig 1. *Change in TMX concentration-effect curves in the presence of different E2 concentrations.*

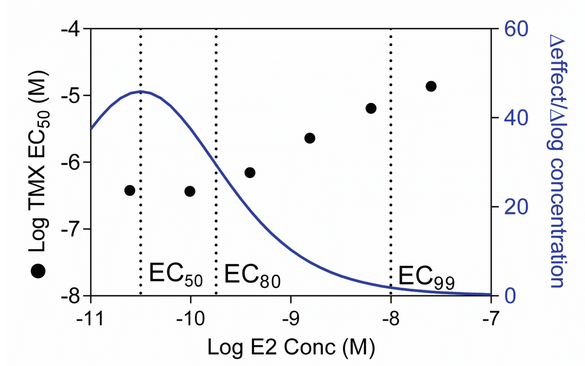

Fig 2. *Changing TMX EC_{50} values with different E2 agonist concentrations (black symbols), with the ratio of change in effect as a function of concentration (blue line).*

Influence of DOC on the ER assay

- HA alone did not have any agonistic effect in the ER assay.
- When HA was added at concentrations expected to cause 20-60% binding the concentration-effect curve of E2 shifted to higher concentrations (Fig 3), increasing the EC_{50} values by a factor of 2.
- The shift was within the usual variability associated with concentration-effect curves between different assay runs.
- The shift was not due to DOC being a non-competitive antagonist or interfering with the assay measurement.

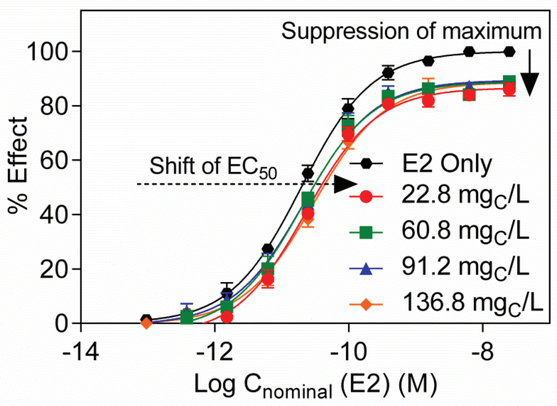

Fig 3. *Nominal E2 concentration-effect curves with HA at concentrations expected to cause 20 to 60% binding.*

Impact of 17β-estradiol sorption to DOC

- To assess if the shift in the E2 concentration-effect curve was due to DOC reducing E2 bioavailability, $C_{DOC\text{-}sorption\ corrected}$ (E2) was predicted based on K_{DOC} values.
- No shift in the EC_{50} values was observed when concentration-effect curves were plotted as $C_{DOC\text{-}sorption\ corrected}$ (E2) (Fig 4).
- This indicates that the increase in nominal EC_{50} value in the presence of HA was due to E2 binding to HA, which reduced its bioavailability.

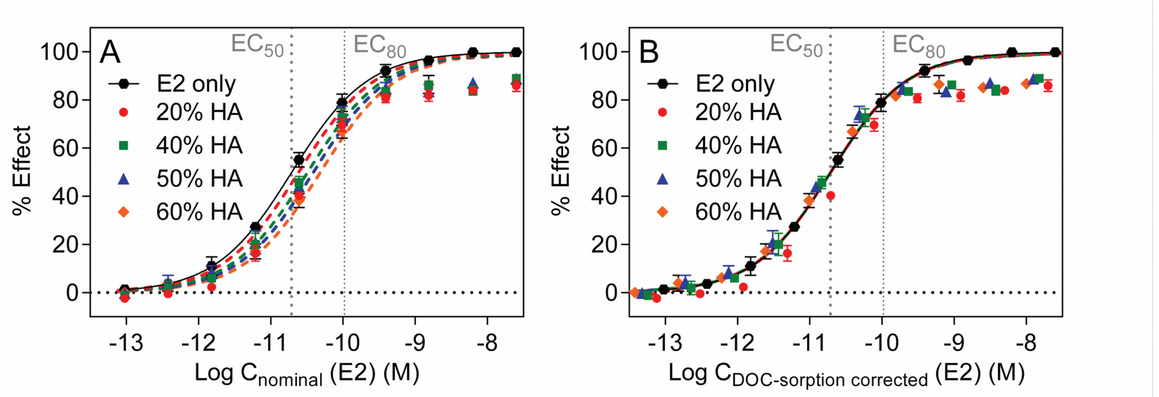

Fig 4. *Experimental (symbols) and modelled (dashed lines) concentration-effect curves for both (A) $C_{nominal}$ (E2) and (B) $C_{DOC\text{-}sorption\ corrected}$ (E2) concentrations.*

Implication of DOC when assessing antagonism

- Nominal agonist concentrations ranging from EC_{50} to EC_{100} are used when assessing antagonism, but sorption to DOC can reduce the nominal concentration, with percent effect of the nominal EC_{50} and EC_{80} agonist concentrations decreasing with increasing DOC concentration (Fig 5A).
- A cut-off of 20% suppression is often set when assessing antagonism. Up to 35% suppression was observed at high HA concentrations, with relative suppression more pronounced at EC_{50} compared to EC_{80} (Fig 5B).
- DOC co-extracted in environmental samples, such as wastewater, can be present at high concentrations in the assay (over 100 mg_C/L), meaning that the samples may be incorrectly reported as 'antagonistic', particularly if an EC_{50} agonist concentration is used. The effect would not be as marked if EC_{80} concentration was used.

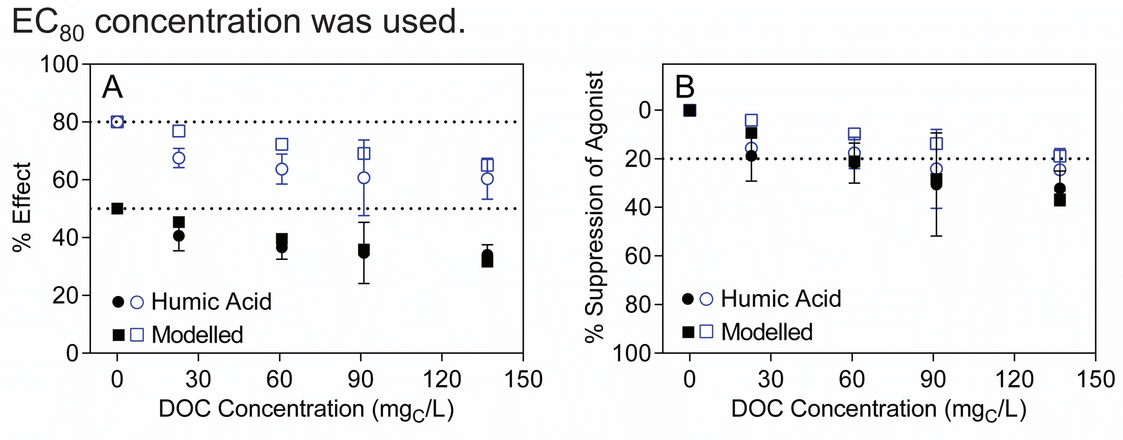

Fig 5. *Experimental and modelled (A) percent effect and (B) percent suppression of the nominal agonist concentration at EC_{50} (black, closed symbols) and EC_{80} (blue, open symbols) as a function of HA concentration.*

Conclusions: EC_{80} agonist concentration is recommended when assessing antagonism *in vitro* to optimise assay sensitivity and reproducibility and to limit the potential influence of co-extracted DOC

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