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Does co-extracted dissolved organic carbon cause artefacts in cell-based bioassays?

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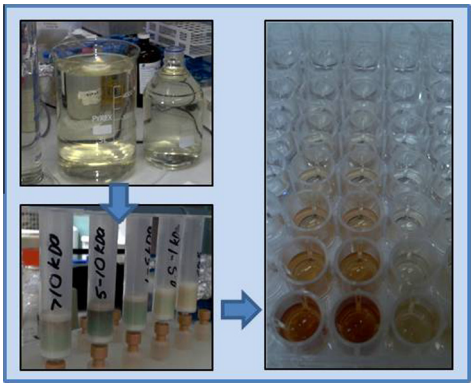
HIGHLIGHTS

- Mixture experiments were used to assess the effect of DOC on cell-based bioassays.
- Studied assays included bacteria, algae and human cell line assays.
- Co-extracted DOC did not cause suppression in the studied cell-based bioassays.
- Low molecular weight DOC may be available in non-specific *Vibrio fischeri* assay.

GRAPHICAL ABSTRACT



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ABSTRACT

Bioanalytical tools are increasingly being employed for water quality monitoring, with applications including samples that are rich in natural organic matter (or dissolved organic carbon, DOC), such as wastewater. While issues associated with co-extracted DOC have been identified for chemical analysis and for bioassays with isolated enzymes, little is known about its effect on cell-based bioassays. Using mixture experiments as diagnostic tools, this study aims to assess whether different molecular weight fractions of wastewater-derived DOC adversely affect cell-based bioassays, specifically the bioluminescence inhibition test with the bacteria *Vibrio fischeri*, the combined algae assay with *Pseudokirchneriella subcapitata* and the human cell line AREc32 assay for oxidative stress. DOC did not cause suppressive effects in mixtures with reference compounds. Binary mixtures further indicated that co-extracted DOC did not disturb cell-based bioassays, while slight deviations from toxicity predictions for low molecular weight fractions may be partially due to the availability of natural components to *V. fischeri*, in addition to organic micropollutants.

1. Introduction

Water scarcity and an increasingly urbanised population have placed a strain on conventional water sources such as dams and groundwater. Consequently, there is a move towards recycling treated wastewater effluent for both non-potable and (indirect) potable applications. Given the incomplete removal of many organic micropollutants (e.g. pesticides, pharmaceuticals, industrial compounds) by conventional wastewater treatment processes (Ying et al., 2009; Singer et al., 2010), an understanding of micropollutant fate during the advanced water treatment processes used for water recycling is essential. While chemical analysis can be

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applied to quantify known micropollutants, it is unable to detect unknowns and transformation products or account for the possible interactions between the many micropollutants present in water. Bioanalytical tools are frequently applied for water quality monitoring complementary to chemical analysis in water treatment plants (e.g. Muller et al., 2008; Macova et al., 2010; Escher et al., 2011; Allinson et al., 2012). *In vitro* bioassays can target all chemicals in a sample that act by the same mode of toxic action, such as photosynthesis inhibition or induction of oxidative stress (Escher and Leusch, 2012). Further, the response in the bioassay is risk-scaled, meaning that more potent micropollutants will elicit a greater effect in the assay than the same concentration of less potent micropollutants.

Municipal wastewater can contain countless different micropollutants at low concentrations. While the concentrations of some chemicals may be below the detection limit of chemical analysis, these micropollutants may act together as a mixture (Silva et al., 2002). Chemicals that share the same mode of action are expected to act according to concentration addition (CA), while chemicals with different modes of action may act by independent action (IA) (Backhaus and Faust, 2012). Mixture experiments can be applied as diagnostic tools to assess the mode of action of different chemicals or environmental samples, as well as potential matrix effects. For example, Neuwoehner et al. (2010) previously used mixture experiments to evaluate the risk potential of transformation products compared to their parent compounds.

Treated wastewater effluent can contain high levels of dissolved organic carbon (DOC), with DOC operationally defined as organic carbon that passes through a 0.45 μm filter. Effluent derived DOC is highly complex and contains natural organic matter and soluble microbial products, in additional to micropollutants (Shon et al., 2006). Solid phase extraction (SPE) is typically applied to extract and enrich micropollutants prior to chemical and bioanalytical testing and a significant fraction of DOC can also be co-extracted in this process (Pichon et al., 1996). While pre-treatment steps to reduce DOC concentration, such as anion exchange, may be suitable when targeting known chemicals (e.g. Blackwell et al., 2004), bioassays targeting unknown chemicals in complex samples require an extraction sorbent suitable for a wide range of chemical properties, meaning that co-extraction of DOC cannot be avoided.

Co-extracted DOC from different origins, including wastewater effluent, has been shown to adversely affect the enzymatic acetylcholinesterase (AChE) inhibition assay even at low DOC concentrations (Neale and Escher, 2013). Tang et al. (2012) also observed growth stimulation of *E. coli* in an assay indicative of protein damage when SPE extracts with high DOC concentrations were tested. Further, reference humic substances, which are not enriched by SPE, can activate receptors (Bittner et al., 2011), suppress androgenic effects of reference compounds (Bittner et al., 2012) and alter reproduction and transcriptional responses in nematodes (Menzel et al., 2005). These studies have focused on whole samples, but DOC can contain a wide range of molecular weight fractions, including biopolymers, humic substances and low molecular weight compounds, which may have a different influence on bioassays.

DOC has the potential to affect bioassays in several ways. Firstly, DOC may modulate micropollutant concentration in the assay. For example Janosek et al. (2007), suggested that antiestrogenic activity observed in the presence of certain humic substances may be due to sorption of the reference compound estradiol, reducing its availability in the assay. Secondly, spectral properties of DOC, such as UV absorbance, have the potential to interfere with the bioassay measurement, with Schreiber et al. (2007) finding that the presence of humic acid altered the light intensity in pulse-amplitude-modulation (PAM) fluorometry. Finally, DOC itself, particularly low molecular weight (<0.5 kDa) fractions, may have an effect on the assay. It has been hypothesised that low molecular weight assimilable organic carbon (AOC) can interfere with the bacterial bioluminescence inhibition test (Macova et al., 2010). *Vibrio fischeri*, which is used in the bioluminescence inhibition test, is able to metabolise AOC and has recently been applied to determine AOC levels in seawater (Jeong et al., 2013). While the SPE enrichment process will alter the DOC properties compared to the raw water, an understanding of the effects of co-extracted DOC is important given that it is routinely applied in bioassays, in contrast to reference humic substances.

Consequently, the current study aims to understand if particular molecular weight fractions of co-extracted wastewater-derived DOC adversely affect commonly used *in vitro* bioassays. The validation of cell-based bioassays is essential if these tools are to be used for water quality monitoring purposes, as is currently proposed. The studied bioassays included the bioluminescence inhibition test with *V. fischeri* as an indicator of baseline toxicity, the combined algae test with photosynthesis inhibition and growth endpoints and the mammalian AREc32 assay for oxidative stress. A multistage ultrafiltration process was applied to fractionate the samples. Ultrafiltration was selected over preparative size exclusion chromatography as it does not require additional eluents and the risk of microbial contamination of the fractions is lower (Muller and Frimmel, 2002). The organic carbon properties of the unfractionated and fractionated samples were assessed, while SPE extraction efficiency of DOC was also determined. Mixture experiments were used as diagnostic tools to assess whether co-extracted DOC impacts the studied cell-based bioassays.

2. Materials and methods

2.1. Sample collection

Water was collected throughout the treatment train of an advanced water treatment plant (AWTP) that utilises membrane filtration and advanced oxidation to treat wastewater effluent in South East Queensland, Australia, from September to November, 2012. Samples were collected from the AWTP inlet (inlet), after microfiltration (MF) and reverse osmosis concentrate (ROC). After collection, the free chlorine concentration was determined and the samples were quenched with sodium thiosulphate, while samples for AOC analysis were quenched with sodium nitrite as sodium thiosulphate can promote bacterial growth, leading to overestimation of AOC (Hammes and Egli, 2007). Chlorine was quenched for preservation of the samples and, in the case of the AOC assay, to allow bacterial growth. Widely used Suwannee River humic acid (HA) (2S101H) and fulvic acid (FA) (2S101F) standards were selected as reference DOC (International Humic Substance Society, St. Paul, US).

2.2. Ultrafiltration fractionation

The water was fractionated into different molecular weight fractions using a stirred cell (Amicon Model 8400, Merck-Millipore, Billerica, US) with membranes with molecular weight cut-offs (MWCO) of 0.5, 1, 5 and 10 kDa (YC and PL series, Merck-Millipore, Billerica, US). The pressure was set to 4 bar using compressed nitrogen gas. Between uses the membranes were stored in 0.5% sodium metabisulphite and thoroughly washed with deionised water with a resistivity of 18.2 MΩ cm^{-1} (MilliQ grade) before fractionating. The samples were fractionated by passing through membranes of decreasing MWCO, with 75 mL of retentate collected for each membrane, while the permeate was fractionated by the next membrane in series. Unfractionated water was also collected to compare with the fractionated samples. With the exception of the

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