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Impact of natural organic matter in water on *in vitro* bioactivity assays

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HIGHLIGHTS

- Fulvic acid and natural organic matter were studied in a panel of bioassays.
- Aryl hydrocarbon receptor activity was induced in a concentration-dependent manner.
- Induced oxidative stress and androgen activity were inhibited.
- Natural organic matter in water has an impact on *in vitro* bioassays.

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ABSTRACT

Surface water can be contaminated with pollutants from multiple sources and contain a vast number of various chemicals. *In vitro* bioassays are valuable tools to assess the total bioactivity of micropollutants in water samples. Besides anthropogenic chemicals, natural organic matter (NOM) is ubiquitous in water, which also may have an impact on the bioactivity in water samples.

In the present study we investigated concentration-dependent effects of Nordic Aquatic fulvic acid (NA-FA) and Nordic reservoir NOM (NR-NOM) on bioactivity measured by a panel of luciferase reporter gene assays. The assays included measurements of both induction of activities and inhibition of induced activation on aryl hydrocarbon receptor (AhR), androgen receptor (AR), estrogen receptor (ER), peroxisome proliferator-activated receptor alpha, and on the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activity as a marker of oxidative stress.

At non-cytotoxic concentrations both NA-FA and NR-NOM induced AhR activity, inhibited AR activity with and without the known inducer dihydrotestosterone, inhibited Nrf2 activity, and NR-NOM induced ER activity. The results indicate that the presence of NOM in water samples may lead to false positive results for AhR activity and false positive results for AR and Nrf2 activity, when assessing inhibition of induced bioactivities from anthropogenic substances. We have demonstrated that NA-FA and NR-NOM have an impact on *in vitro* bioactivities and conclude that the impact of NOM in water should be considered in the evaluation of results from bioactivity assays.

1. Introduction

Surface water is used for drinking water production in many areas and is prone to contamination from multiple sources (Houtman, 2010). Water contaminants encompass a wide range of substances such as chemicals from agriculture, industry, and consumer products (Houtman, 2010). Due to the chemical complexity of surface water and limited data on toxicity for individual and combinations of chemicals, assessment of potential adverse effects on the ecosystem and humans is a challenge. *In vitro* bioassays are now increasingly used to examine various waters (Conley et al., 2017a, 2017b; Escher et al., 2014; Välitalo et al., 2017), as they allow assessment of the total bioactivity of all chemicals present.

In addition to anthropogenic chemicals, natural organic matter (NOM) constitutes a key component of surface water (Sillanpää, 2015). NOM is the complex mixture of aquatic organic materials, which stem from breakdown products of terrestrial plants and by-products of algae, bacteria and plants in the water body itself (Sillanpää, 2015). The characteristics of NOM vary depending on e.g. geographical location (Fabris et al., 2008), season (Wei et al., 2008), and water source origin (Owen et al., 1995). Thus, NOM consists of

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heterogeneous mixtures of compounds varying in e.g. chemical charge, chemical composition, molecular size, and molecular weight encompassing functional groups such as esteric-, phenolic-, carboxylic-, quinine-, amino-, and nitroso-groups (Sillanpää, 2015). NOM in rivers and lakes is mainly present as dissolved organic carbon (DOC) including hydrophobic and hydrophilic components (Schumacher et al., 2006). The hydrophobic acids, also called humic substances, constitute approximately 50% of the total organic carbon in water (Sillanpää, 2015; Świetlik et al., 2004). Humic substances consist mainly of fulvic acids (FAs) and smaller amounts of humic acids (HAs) and humins, where FAs are more water soluble than HAs (Sillanpää, 2015).

In vitro bioassays are valuable tools in water quality assessment. A set of responsive and relevant bioassays for testing of water samples has been proposed, covering xenobiotic metabolism (aryl hydrocarbon receptor, AhR, peroxisome proliferator-activated receptor, PPAR), hormone-mediated modes of action (estrogen receptor, ER, androgen receptor, AR and glucocorticoid receptor), and reactive modes of action (genotoxicity and oxidative stress) (Escher et al., 2014). The activities can be measured both in agonist and in antagonist modes. Thus, for determination of antiestrogenic and antiandrogenic effects, activities are initially induced by a positive control, 17β-estradiol and dihydrotestosterone, respectively, and the inhibition of the induced activity by the test sample is determined.

Due to the ubiquitous presence of NOM in water resources, it is important to understand how these may affect *in vitro* bioactivity of anthropogenic chemicals in water samples, especially since DOCs are known to be extracted to some extent by the commonly used solid phase extraction (SPE) procedure of water samples (Neale and Escher, 2014). NOM may affect *in vitro* bioactivity either 1) directly by activation or inhibition of the biological pathway of interest or 2) indirectly through binding of positive controls or micropollutants in the sample. Both types of interaction may lead to false positive or false negative results in studies where the main objective is to determine the toxic potential of micropollutants in water.

In this study, we investigated the effects of Nordic Aquatic Fulvic acid (NA-FA) and Nordic Reservoir Natural Organic Matter (NR-NOM) in a battery of *in vitro* bioassays commonly used for water quality assessment, including induction/inhibition of AhR, AR, ER, and PPARα receptors, as well as oxidative stress (nuclear factor erythroid 2-related factor 2, Nrf2). Bioactivities were observed on all endpoints except on PPARα activity and the results suggest that NOM in water needs to be considered in the assessment of *in vitro* bioassay data.

2. Materials & methods

2.1. Test substances

Nordic Aquatic Fulvic Acid (NA-FA) (1R105F) and Nordic Reservoir Natural Organic Matter (NR-NOM) (1R108 N) were purchased from the International Humic Substance Society (IHSS) (St Paul, USA). According to IHSS, the elemental carbon content was 52.31 and 53.17% (w/w) for NA-FA and NR-NOM, respectively. The powder was stored in darkness at room temperature. Stocks were prepared in a concentration of 60 g L^{-1} in autoclaved 0.05 M sodium hydroxide (NaOH) and stored for a maximum of 3 weeks in darkness at 4 °C.

Positive controls (all purchased from Sigma-Aldrich) including WY-14,643 (WY), 2,3,7,8-tetrachlorodibenzodioxin (TCDD), 17β-estradiol (E2), raloxifene HCl, dihydrotestosterone (DHT), hydroxyflutamide (OHF), and sulforaphane (SFN) were prepared in ethanol or DMSO.

2.2. Cell culture

The human hepatocellular carcinoma (HepG2) cell-line was cultured in high glucose content DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Lonza), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} Gibco amphotericin B (Gibco). Experimental medium is equivalent to culture medium for reporter gene assays conducted in HepG2 cells.

The human breast carcinoma cell-line (VM7Luc4E2, donated by Professor Michael Denison, University of California, USA) was cultured in RPMI 1640 (Gibco) supplemented with 8% FBS (Gibco), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Lonza), and 0.55 mg mL^{-1} gentamicin (Gentamicin Sulfate, Lonza). Two to three days prior to experimental set-up, cells were transferred to estrogen free medium consisting of high-glucose content DMEM (Lonza), 4.5% dextran-charcoal treated FBS (Thermo Scientific), 4 mM L-glutamine (Lonza), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Lonza), and 0.38 mg mL^{-1} gentamicin (Lonza). The reporter gene assay was performed in experimental medium consisting of estrogen free medium without addition of gentamicin.

The Chinese Hamster Ovary cell-line (AR-EcoScreen™, National Institutes of Biomedical Innovation, Health, and Nutrition JCRB cell bank) was cultured in DMEM F12 medium (Sigma) supplemented with 5% FBS (Gibco), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, (Lonza), 2 mM L-glutamine (Lonza), 100 μg mL^{-1} hygromycin B (Invivogen), and 200 μg mL^{-1} zeocin (Invitrogen). The reporter gene assay was conducted in experimental medium with FBS exchanged for dextran-charcoal treated FBS (Thermo Scientific) and without hygromycin and zeocin.

Cells were cultured in a humidified environment at 37 °C and 5% CO_2 and medium was exchanged every 2–3 days.

2.3. Cell treatment

On the treatment day, serial dilutions of NA-FA and NR-NOM were prepared in 0.05 M NaOH from the 60 g L^{-1} stock. These were diluted in experimental medium to a concentration of 2× or 10× the final concentration, depending on reporter gene assay. Medium was completely removed from cell culture wells and new experimental medium was added to each well together with the 2× or 10× concentrated NA-FA and NR-NOM, to give a final concentration of 1×. Controls were prepared in a similar manner and vehicle concentrations were 250 μM NaOH in treatment groups and controls.

Cell viability of NA-FA and NR-NOM in concentrations from 100 to 300 mg L^{-1} in HepG2 cells and from 20 to 200 mg L^{-1} in VM7Luc4E2 and AR-EcoScreen™ cells was tested. In the reporter gene assays, cells were treated with NA-FA and NR-NOM in concentrations of 5–200 mg L^{-1}. After adjusting for ash content of 0.45% and 41.5% and elemental carbon content of 52% and 53% for NA-FA and NR-NOM, respectively, these concentrations (5–300 mg L^{-1}) correspond to 2.6–156.2 mg_C L^{-1} and 1.6–93.2 mg_C L^{-1}, respectively.

Positive controls were included when NA-FA and NR-NOM were tested alone for ability to induce reporter activity: WY at 10 μM, SFN at 6.25 μM, DHT in concentrations of 0.001–1000 nM, and E2 in concentrations of 0.04–367 pM. DHT and E2 were tested in a concentration range, according to the OECD guidelines (OECD, 2016, 2012). Antagonistic activity of NA-FA and NR-NOM were tested in combination with a constant concentration of a positive control. Here, the concentrations of positive controls were 10 μM WY, 1000 pM TCDD, 3.13 μM SFN, 500 pM DHT, and 92 pM E2 in the PPARα, AhR, Nrf2, AR, and ER reporter gene assays, respectively. The concentrations correspond approximately to EC_{50} to EC_{80},