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Interlaboratory comparison of *in vitro* bioassays for screening of endocrine active chemicals in recycled water

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ABSTRACT

In vitro bioassays have shown promise as water quality monitoring tools. In this study, four commercially available *in vitro* bioassays (GeneBLAzer® androgen receptor (AR), estrogen receptor-alpha (ER), glucocorticoid receptor (GR) and progesterone receptor (PR) assays) were adapted to screen for endocrine active chemicals in samples from two recycled water plants. The standardized protocols were used in an interlaboratory comparison exercise to evaluate the reproducibility of *in vitro* bioassay results. Key performance criteria were successfully achieved, including low background response, standardized calibration parameters and high intra-laboratory precision. Only two datasets were excluded due to poor calibration performance. Good interlaboratory reproducibility was observed for GR bioassay, with 16–26% variability among the laboratories. ER and PR bioactivity was measured near the bioassay limit of detection and showed more variability (21–54%), although interlaboratory agreement remained comparable to that of conventional analytical methods. AR bioassay showed no activity for any of the samples analyzed. Our results indicate that ER, GR and PR, were capable of screening for different water quality, *i.e.*, the highest bioactivity was observed in the plant influent, which also contained the highest concentrations of endocrine active chemicals measured by LC–MS/MS. After advanced treatment (*e.g.*, reverse osmosis), bioactivity and target chemical concentrations were both below limits of detection. Comparison of bioassay and chemical equivalent concentrations revealed that targeted chemicals accounted for ≤5% of bioassay activity, suggesting that detection limits by LC–MS/MS for some chemicals were insufficient and/or other bioactive compounds were present in these samples. Our study demonstrated that *in vitro* bioassays responses were reproducible, and can provide information to complement conventional analytical methods for a more comprehensive water quality assessment.

1. Introduction

The scarcity of potable water is a growing issue worldwide, particularly for urban centers located in arid regions. To fulfill water supply needs, impacted entities are pursuing policies to increase the supply and use of recycled water (SWRCB, 2013). Herein we define recycled water as highly treated municipal wastewater that is available for indirect potable reuse (NRMMC, 2009), and ultimately in the future for direct potable reuse (WRRF, 2011). Because treated wastewater effluents that serve as source water for recycled water facilities typically contain chemical residues (Ternes et al., 2004), purification is needed to attenuate these contaminants. Prior to widespread public acceptance of potable reuse, recycled water utilities are faced with the challenge of demonstrating that chemicals present in product water are not harmful to environmental and human health (WRRF, 2011; SWRCB, 2013).

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Bioanalytical techniques such as *in vitro* bioassays have been shown to be a suitable screening alternative for water quality applications. Many of these assays are used to quantify chemical bioactivity based on mode of action (MOA), *e.g.*, as part of the U.S. Environmental Protection Agency (EPA) ToxCast™ and Endocrine Disruptor Screening Programs (Dix et al., 2007; Reif et al., 2010). Over the last decade, a number of studies have applied *in vitro* bioassays to ascertain the endocrine activity of surface water and wastewater (van der Linden et al., 2008; Leusch et al., 2010; Jarosova et al., 2014). Moreover, bioassays that target molecular initiating events (*e.g.* gene transactivation) can be linked to higher order adverse outcomes via toxicity pathway analyses (Piersma et al., 2013; Sonneveld et al., 2006), providing additional biological relevance for these tools in the screening mode. Whereas much of the groundwork has been laid for endocrine disrupting endpoints, researchers are currently broadening the scope of bioanalytical tools to include other relevant MOAs, *e.g.*, genotoxicity, immunotoxicity and oxidative stress (Escher et al., 2014; Leusch et al., 2014; van der Linden et al., 2014).

A recent study by Escher et al. (2014) evaluating 103 different *in vitro* bioassays to screen for chemicals in wastewater, recycled water and drinking water, concluded that some of the bioassay endpoints were capable of discriminating among samples of different quality. Other studies have reached a similar conclusion, showing that a few bioassays are capable of relatively good measurement precision (within laboratories) while demonstrating the ability to differentiate among water qualities (Leusch et al., 2010; Jarosova et al., 2014). However, these studies have employed bioassays that are not widely available, and as a result, lack standardization across multiple laboratories. To successfully transfer and implement this technology for water quality monitoring, it is critical to demonstrate that commercially available bioassays can be standardized, and that measurements using these standardized assays agree well across multiple laboratories (Andersen et al., 1999).

The goals of this study were to 1) develop standardized protocols using commercially available bioassays for screening water samples from recycled water treatment process units; 2) evaluate the reproducibility of the bioassay responses among participating laboratories; and 3) assess the ability of the bioassays to screen for water quality by comparing bioassay responses with targeted chemical occurrence. To accomplish our goals, four "off-the-shelf" *in vitro* transactivation bioassays representing different pathways of the endocrine system were adapted to screen water samples from two water recycling plants. Replicate aliquots of water extracts were analyzed by five research laboratories using the standardized protocols, with bioassay responses translated into bioanalytical equivalent concentrations and compared with conventional analytical measurements.

2. Materials and methods

2.1. Materials

GeneBLAzer® androgen receptor (AR), estrogen receptor-alpha (ER), glucocorticoid receptor (GR) and progesterone receptor (PR) cell assay kits and media components were purchased from Life Technologies (Carlsbad, CA). Bioassay kits contained division arrested cells stably transfected with the beta-lactamase reporter gene, a LiveBLAzer FRET B/G loading kit, and CCF4-AM substrate. Black wall, clear-bottom 96-well plates were purchased from Corning (Corning, NY).

Chemicals known or suspected to activate AR (testosterone, trenbolone), ER (17α-ethinylestradiol, 17α-estradiol, 17β-estradiol, bisphenol A, estriol, estrone), GR (dexamethasone, hydrocortisone, prednisolone, and triamcinolone), and PR (levonorgestrel, norethisterone, norgestrel) were purchased at the highest purity available from Sigma–Aldrich (St. Louis, MO). The AR active chemical methyltrienolone (R1881) was purchased from Perkin–Elmer. Isotopically labeled 17α-ethinylestradiol-$^{13}C_2$, bisphenol A-$^{13}C_{12}$, estriol-$^{13}C_3$, estrone-$^{13}C_6$ were purchased from Cambridge Isotope Laboratories (Andover, MA), dexamethasone-d_4, norethindrone-d_6 and norgestrel-d_6 from C/D/N Isotopes (Pointe-Claire, Canada), and 17β-estradiol-$13C_3$ and hydrocortisone-d_2 from Sigma–Aldrich (St. Louis, MO). The purity of all the isotope standards was ≥98%.

Molecular grade dimethyl sulfoxide (DMSO, 99.5% purity) was obtained from Sigma–Aldrich (St. Louis, MO). HPLC grade methanol, acetone, acetonitrile, ethyl acetate, hexane and formic acid were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Sample collection and processing

Grab samples of water (4 L each) were collected in June 2013 from various treatment processes of a fully operational (Plant 1) and a pilot water recycling plant (Plant 2) located in southwestern U.S (Table 1). Upon collection, samples containing chlorine were immediately quenched with sodium thiosulfate (50 mg/L). All water samples and a field blank sample consisting of milli-Q water were treated with sodium azide (1 g/L) to inhibit microbial activity (Vanderford et al., 2011). Samples were stored in methanol-rinsed amber glass bottles at 4 °C and extracted within one week of collection.

Solid phase extraction (SPE) was performed according to the methods described by Macova et al. (2011). Briefly, 1 L samples were filtered and passed through two pre-conditioned cartridges: a 6 cc Oasis HLB cartridge (Waters, Milford, MA) stacked on top of a Supelclean coconut charcoal cartridge (Sigma Aldrich). After rinsing with milli-Q water and vacuum drying for 2 h, the cartridges were eluted separately with 10 mL methanol and 10 mL acetone:hexane (1:1, v/v). For each 4-L water sample, all eluates were combined and evaporated under a gentle stream of nitrogen. Each water extract was then reconstituted in 4 mL methanol and aliquots of 1.5 mL were stored at −20 °C for chemical analyses. The remaining extract was solvent exchanged to 2.5 mL DMSO and kept in amber glass vials at −20 °C. Aliquot samples of 500 μL were shipped in plastic Eppendorf tubes on ice overnight to the participating laboratories for blind bioassay analysis, where they were transferred into amber glass vials and stored at −20 °C.

Table 1
Samples collected from two U.S. water recycling treatment plants. Plant 1 was a fully operational facility; Plant 2 was operating as a pilot plant at the time of collection.

Sample no.	Description
1	Field blank (milli-Q water)
2	Plant 1 – influent (final secondary effluent from WWTP#1)
3	Plant 1 – influent subject to ozonation (Oz)
4	Plant 1 – product water subject to microfiltration (MF)
5	Plant 1 – product water subject to reverse osmosis (RO)
6	Plant 1 – product water subject to ultraviolet (UV)
7	Plant 2 – influent (final secondary effluent from WWTP#2)
8	Plant 2 – influent subject to UV
9	Plant 2 – influent subject to UV/hydrogen peroxide (H_2O_2)
10	Plant 2 – influent subject to Oz
11	Plant 2 – influent subject to Oz/UV
12	Plant 2 – influent subject to chlorination

WWTP – wastewater treatment plant.