Exploring the oxidative stress response mechanism triggered by environmental water samples

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Abstract

Environmental waters can contain a wide range of micropollutants. Bioanalytical test batteries using assays indicative of different stages of cellular toxicity pathways, such as adaptive stress responses, have been applied to a range of water samples. Oxidative stress response assays have proven to be sensitive tools, but the mechanism by which water samples are inducing the oxidative stress response remains unclear because both electrophiles and reactive oxygen species (ROS) may activate the Nrf2-antioxidant response element (ARE) pathway. The current study aimed to explore the underlying mechanisms of the oxidative stress response triggered by exposure to surface water extracts previously shown to be active in the ARE GeneBLAzer oxidative stress response assay.

ROS formation and changes in glutathione (GSH) concentration were assessed in human liver cells exposed to water extracts from a large river in addition to individual chemicals that were detected in these water extracts and reported to be active in the ARE GeneBLAzer assay in a previous study. Many of the surface water samples induced ROS formation and decreased the GSH to glutathione disulfide (GSSG) ratio, suggesting that the formation of ROS is an important mechanism. However, some of the most responsive samples in the ARE GeneBLAzer assay, as well as the individual chemicals, did not have a significant effect on either ROS formation or the GSH/GSSG ratio, suggesting a different mechanism. Antioxidants can also induce the Nrf2-ARE pathway and the ARE GeneBLAzer assay may also detect compounds that activate ARE by Nrf2-independent mechanisms, thus further research is required to characterise active chemicals in oxidative stress response assays. However, these tests are still useful for quantifying the integrated cellular response to multiple molecular initiating events and can be used complementary to assays indicative of specific effects, such as receptor-mediated assays.
Introduction

The aquatic environment can contain a diverse range of organic micropollutants and exposure to these contaminants can potentially have implications for both ecological and human health\(^1,2\). Water quality monitoring typically utilises targeted chemical analysis. However, because of the wide range of contaminants and their transformation products in the environment, targeted chemical analysis can only provide partial information. As a result, *in vitro* bioanalytical tools are applied alongside to provide information about the effect of all chemicals in a sample.\(^3\) A recent study that applied over 100 *in vitro* bioassays to a range of water samples found that oxidative stress response assays were among the most responsive assays tested.\(^4\) The oxidative stress response is an important cellular adaptive stress response pathway and is typically activated at lower chemical concentrations than those that cause cell death.\(^5\) *In vitro* bioassays indicative of the oxidative stress response, such as AREc32 and ARE GeneBLAzer, have been applied to individual chemicals,\(^6-8\) chemical mixtures\(^9\) and environmental samples, including drinking water, surface water and wastewater.\(^10-12\) The oxidative stress response assays have proven to be sensitive tools, but mixture toxicity modelling studies have shown that detected chemicals can only explain a small fraction (<2%) of the observed oxidative stress response in water samples.\(^9,13\)

The above mentioned oxidative stress response assays, ARE GeneBLAzer and AREc32, contain beta-lactamase or luciferase reporter genes, respectively, paired to the stable antioxidant response element (ARE).\(^14,15\) AREs are present in the regulatory region of genes involved in detoxification and the oxidative stress response and are activated by the transcription factor Nrf2. Under basal conditions, Nrf2 is repressed in the cytoplasm by negative regulator Keap1, but in the presence of oxidative stress or electrophilic chemicals, Nrf2 is released from Keap1 and translocates to the nucleus, where it forms heterodimers with Maf proteins and activates ARE, resulting in antioxidant gene expression.\(^16-18\) Keap1 contains many cysteine residues, and electrophiles and reactive oxygen species (ROS) can modify reactive cysteine residues through oxidation, alkylation or reduction of the sulfhydryl group, changing the conformation of Keap1 and leading to the release of Nrf2.\(^19-21\) While the oxidative stress response assays indicate the presence of chemicals inducing Nrf2 activation, the assays do not provide information about whether the chemicals are inducing the Nrf2 pathway due to the presence of reactive oxygen species (ROS) or electrophilic compounds.

Not all electrophiles will react with the sulfhydryl group. Electrophiles can be classified as hard or soft based on their molecular properties, with soft electrophiles typically large, highly polarizable molecules, while hard electrophiles are smaller molecules with low polarizability.\(^22\) Biological nucleophiles can also be classified as hard, such as DNA, or soft, such as the sulfhydryl group of cysteine residues, based on their polarizability and electrophiles prefer to react with nucleophiles of a similar softness or hardness.\(^23,24\) Thus soft electrophiles, which include many
disinfection by-products, the biocide Sea-Nine and the industrial compound ethyl acrylate can be expected to activate the oxidative stress response via the Nrf2-ARE pathway.

Tripeptide γ-L-glutamyl-L-cysteinyl-glycine or glutathione (GSH) is a soft nucleophile and plays an important protective role in the cell against ROS and electrophilic chemicals. GSH can act as an antioxidant to reduce ROS, leading to the formation of glutathione disulfide (GSSG), and can react with soft electrophiles to form GSH conjugates, which is often catalysed by glutathione-S-transferase (GST) enzymes. Alterations in GSH concentrations are indicative of cellular stress, with increased levels of GSSG suggestive of oxidative stress and a decreased ratio of reduced GSH to GSSG (GSH/GSSG) reflecting changed cellular redox conditions. Consequently, quantifying reduced GSH, GSSG and the GSH/GSSG ratio in exposed cells can provide insights into the mechanisms by which water samples induce oxidative stress. In addition to protecting against electrophiles and ROS, GSH can also have a number of other functions in the cell, including storage of cysteine and regulating cell growth.

In this study, we aimed to gain a better understanding of the mechanisms underlying the oxidative stress response triggered by surface water samples from the Danube River previously reported to be active in the ARE GeneBLAzer assay. This was achieved by assessing ROS formation and changes in glutathione (GSH) concentration in human HepG2 liver cells in vitro, which is the same cell line as used to develop the ARE GeneBLAzer assay. Further, five environmental chemicals that were detected in the Danube River samples and are known to be active in the ARE GeneBLAzer assay were also tested. The five chemicals represent different use groups and include the industrial compound 2,4-dinitrophenol, the food additive caffeine, the pharmaceutical diclofenac, the phytoestrogen genistein and the pesticide metolachlor.

Materials and Methods

Materials

2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Sigma Aldrich (Castle Hill, Australia). DCFH-DA stock solution of 10 mM was prepared in methanol on the day of the experiment, with powdered DCFH-DA stored under nitrogen at -20°C. The GSH/GSSG-Glo assay kit was purchased from Promega (Alexandria, Australia). GSH/GSSG-Glo assay positive control menadione was purchased from Sigma Aldrich (Castle Hill, Australia), while DCFH-DA assay positive control 30% (w/w) hydrogen peroxide (H₂O₂) was purchased from Chem-Supply (Gillman, Australia). Environmental chemicals 2,4-dinitrophenol, caffeine, diclofenac, genistein and metolachlor were purchased from Sigma Aldrich (Castle Hill, Australia).
Water samples

Large volume solid phase extraction (LVSPE) water samples from the 2013 Joint Danube Survey (JDS) were applied in this study. The samples have been extensively characterised by both chemical analysis and bioanalysis, with the majority of samples found to induce oxidative stress in the ARE GeneBLAzer assay. Only samples that were active in the ARE GeneBLAzer assay were included in this study. Information about sample enrichment and extraction, as well as sampling locations, can be found in the Supporting Information of Neale et al., with the JDS site numbers referring to the location of the sampling sites along the Danube River. Relative enrichment factors (REF) were used as concentration measures for the water samples and provide an indication of the sample enrichment or dilution required to see an effect in the assay. The REF (Equation 1) was calculated using the sample enrichment factor after LVSPE, which was based on the total volume of water sampled and the final volume of the sample extract (Equation 2), and the assay dilution factor (Equation 3).

\[
\text{REF} = \text{dilution factor}_{\text{assay}} \cdot \text{enrichment factor}_{\text{LVSPE}}
\]

\[
\text{enrichment factor}_{\text{LVSPE}} = \frac{\text{total volume of water}}{\text{volume of extract}}
\]

\[
\text{dilution factor}_{\text{assay}} = \frac{\text{volume of extract added to assay}}{\text{total volume of assay}}
\]

The sample REF for the GSH/GSSG-Glo assay was the effect concentration causing an induction ratio of 1.5 (EC_{IR1.5,ox stress}) in the ARE GeneBLAzer assay, while a three-point dilution series starting at double the EC_{IR1.5,ox stress} was used for samples in the DCFH-DA assay. EC_{IR1.5,ox stress} indicates a 50% increase in effect compared to the negative control and is close to the assay limit of detection. Cell viability was assessed in parallel to induction using resazurin in Neale et al., and cytotoxic concentrations were excluded from the DCFH-DA and GSH/GSSG assays. The five chemicals were analysed in the DCFH-DA and GSH/GSSG-Glo assays at concentrations in the range of their ARE GeneBLAzer EC_{IR1.5,ox stress} values (Table S1). The EC_{IR1.5,ox stress} values were derived using raw emission data from the US EPA ToxCast database, with further information about the data evaluation method found in Neale et al.
**Cell culture**

HepG2 cells (European Collection of Authenticated Cell Cultures, Cat No. 85011430) were used for the DCFH-DA and GSH/GSSG-Glo assays. The cells were grown in DMEM with GlutaMAX™ with 10% dialysed fetal bovine serum (FBS) (Thermo Fisher Scientific, Scoresby, Australia) and passaged every 3 to 4 days.

**DCFH-DA assay**

Intracellular ROS formation was assessed using the DCFH-DA assay, where DCFH-DA is taken up by intact cells and enzymatically hydrolysed to non-fluorescent DCFH by cellular esterases. DCFH is then oxidised to fluorescent DCF in the presence of ROS. The DCFH-DA assay was conducted based on the protocol outlined in Wang and Joseph, with some modifications. Briefly, 100 µL of HepG2 cells in DMEM with GlutaMAX™ were seeded at 2×10⁵ cells/mL in 96 well black clear bottom plates and incubated for 24 h at 37°C, 5% CO₂. After 24 h the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS), then 100 µL of phenol red free DMEM containing 100 µM DCFH-DA was added to the cells. After incubating at 37°C in the dark for 30 min, the medium was removed and the cells were again washed twice with PBS, then 100 µL of water extract or test chemical serially diluted in PBS was added. All samples were prepared in methanol, with the solvent concentration in the assay not exceeding 1% to prevent any solvent effects. H₂O₂ served as the positive control, while PBS only served as the negative control. Fluorescence at 485 and 520 nm was measured using a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany), with readings taken immediately after sample addition (t=0) and at 2 h (t=2). Each sample was run in triplicate per experiment, with each experiment conducted at least twice. Percent fluorescence increase at 2 h was calculated for both the samples and PBS controls using Equation 4, where RFU refers to relative fluorescence units. The fluorescence induction ratio was calculated using Equation 5. Linear concentration-effect curves with an intercept of zero and a fitted slope were used to determine the effect concentration causing a ROS formation induction ratio of 1.5 (EC_{IR1.5,ROS}) (Equation 6).

\[
\%\text{increase } t=2 = \frac{\text{RFU}_{\text{sample or PBS } t=2} - \text{RFU}_{\text{PBS } t=0}}{\text{RFU}_{\text{PBS } t=0}} 
\]

(4)

\[
\text{Induction ratio (IR)} = \frac{\%\text{increase sample } t=2}{\%\text{increase PBS } t=2} 
\]

(5)
EC_{IR1.5,ROS} = \frac{0.5}{\text{Slope}} 

(GSH/GSSG-Glo Assay)

Reduced GSH, GSSG and the GSH/GSSG ratio were determined using the GSH/GSSG-Glo assay, which is based on the conversion of a luciferin derivative GSH probe to luciferin by GST. The GSH/GSSG-Glo assay was conducted according to the Promega technical manual, with some modifications. Briefly, the samples were diluted in Krebs-Henseleit buffer (Section S1 and Table S2 of the Supplementary Information) in a 96 well plate, then 4 µL of pre-diluted sample was added to a white low volume 384 well plate. Each sample had 4 replicates for both total glutathione and GSSG analysis. The final sample concentration in the assay was the EC_{IR1.5,ox stress} values in units of REF or M (Tables 1 and S1). HepG2 cells were washed twice and resuspended in Krebs-Henseleit buffer at a concentration of 2.5×10^5 cells/mL, with 4 µL added to each well, giving a concentration of 1,000 cells/well. The cells were exposed to the samples for 1 h at 37°C, then 2 µL of either Total Glutathione Lysis Reagent (Promega) for total glutathione analysis or Oxidised Glutathione Lysis Reagent (Promega) for GSSG analysis was added for cell lysis and the samples were shaken for 5 min at 500 rpm. After shaking, 2 µL of Luciferin Generation Reagent (Promega) was added to each well and the samples were incubated for 30 min at room temperature. Finally, 8 µL of Luciferin Detection Reagent (Promega) was added to each well and allowed to equilibrate for 15 min at room temperature, and then luminescence in relative light units (RLU) was measured using a Fluostar Omega microplate reader. Menadione served as the positive control and methanol was the solvent control, while cell-free controls were also included. A GSH standard curve from 0.5 to 8 µM in the absence of cells was also included in each experiment to convert RLU to total GSH concentration. GSSG concentration was calculated from the standard curve with the GSH concentration divided by two as one mole of GSSG is equivalent to two moles of GSH in the GSH/GSSG assay. The cell-free RLU was subtracted from the sample RLU. The GSH/GSSG ratio was calculated for each sample using Equation 7, while reduced GSH was calculated by subtracting GSSG from total GSH. Statistically significant changes in reduced GSH, GSSG and GSH/GSSG ratio compared to the solvent control were determined using a two-tailed t-test (p-value < 0.01).

\[
\text{GSH/GSSG ratio} = \frac{\text{Total GSH (µM)} - (2 \times \text{GSSG (µM)})}{\text{GSSG (µM)}}
\]
**Results**

**ROS formation**

Nineteen JDS samples were tested in the DCFH-DA assay, with the concentration-effect curves shown in Figure S1 of the Supplementary Information. The majority of the surface water samples induced ROS formation in the studied concentration range, with the exception of JDS 32, 41, 53, 59, 63 and 65. EC\textsubscript{IR1.5,ROS} values were calculated for the active samples and compared with the ARE GeneBLAzer EC\textsubscript{IR1.5,ox stress} values previously published in Neale et al.\textsuperscript{10} (Figure 1). Most of the active samples induced ROS at lower or similar REFs as induced the oxidative stress response in the ARE GeneBLAzer assay, though JDS 57 and 60 only induced ROS at concentrations higher than the respective ARE GeneBLAzer EC\textsubscript{IR1.5,ox stress} values (Table 1). Five chemicals detected in these water samples and reported to be active in the ARE GeneBLAzer assay were also tested in the DCFH-DA assay at concentrations in the range of their ARE GeneBLAzer EC\textsubscript{IR1.5,ox stress} values (Table S1). None of the chemicals increased ROS formation in the studied concentration range (Figure S2).

**Changes in glutathione concentration**

Reduced GSH and GSSG concentrations, as well as the GSH/GSSG ratio, were determined for the JDS samples using the GSH/GSSG-Glo assay (Figure 2), with all samples tested at the REF inducing EC\textsubscript{IR1.5,ox stress} in the ARE GeneBLAzer assay. Compared to the methanol control, 13 JDS samples (JDS 8, 22, 27, 29, 30, 32, 33, 35, 36, 37, 39, 41 and 44) significantly decreased the reduced GSH concentration in the cells, while 15 samples (JDS 8, 22, 27, 30, 32, 33, 36, 37, 39, 44, 53, 57, 60, 63 and 65) increased the GSSG concentration. Twelve samples, JDS 8, 22, 27, 29, 30, 32, 33, 35, 36, 37, 39 and 44, also significantly decreased the GSH/GSSG ratio. The active samples are summarised in Figure 3.

None of the five studied chemicals depleted the reduced GSH concentration, increased the GSSG concentration or decreased the GSH/GSSG ratio (Figure 4), though metolachlor increased the reduced GSH concentration compared to the control (p-value <0.01).

**Discussion**

Environmental waters, such as surface waters, can contain a wide range of compounds and their transformation products. Of the 7,522 chemicals analysed in the ARE GeneBLAzer assay as part of the US EPA ToxCast program, 23% were reported to be active in the assay.\textsuperscript{33} Consequently, many of the chemicals present in the JDS samples may contribute to the observed oxidative stress response. Of the 92 chemicals detected in the studied JDS samples, 15 were reported to be active in the ARE GeneBLAzer assay, according to the most recent version of the US EPA ToxCast
Database. The five chemicals selected for further study were prioritised based on their frequency of occurrence in the JDS samples (present in 42 to 100% of the studied samples), detected concentration (e.g., caffeine found at concentrations up to 1029 pM) and reported effect in the US EPA ToxCast database. With the exception of metolachlor, which increased the reduced GSH concentration in HepG2 cells, none of the five studied chemicals were responsive in the DCFH-DA or GSH/GSSG-Glo assays. Wu et al. reported increased ROS formation in HepG2 cells in the presence of diclofenac, but only at higher concentrations than tested in the current study. Increased GSH concentration in the presence of metolachlor was also previously observed by Dierickx in HepG2 cells, with the increased GSH concentration attributed to cellular defence mechanisms. In contrast, the phytoestrogen genistein is an antioxidant and was reported to increase Nrf2 expression in Caco-2 cells via protein kinase pathways, leading to activation of the ARE. The mechanisms by which antioxidants can induce the Nrf2-ARE pathway are discussed in further detail below.

The ToxCast database also includes the Attagene Nrf2/ARE-cisFACTORIAL assay using HepG2 cells, but only 2,4-dinitrophenol and metolachlor were reported as active. Further, caffeine, genistein and metolachlor were reported to be inactive in the Aprelica 24 h HepG2 oxidative stress assay (2,4-dinitrophenol and diclofenac were not tested). The ARE GeneBLAzer assay was constructed using a human NAD(P)H:quinone oxidoreductase 1 (NQO1) gene, which has the potential to bind other transcription factors, such as c-Jun and MafK, in addition to Nrf2, with some likely to be positive factors and activate ARE, while others may downregulate expression. Further, the ARE GeneBLAzer assay may be susceptible to false positives due to the assay specifications, such as promoter construction and ARE orientation, which enables ARE GeneBLAzer to potentially detect compounds that activate ARE through non-specific mechanisms, in addition to Nrf2 dependent mechanisms. This may explain why some of the studied chemicals known to be active in the ARE GeneBLAzer assay did not have any effect in the DCFH-DA and GSH/GSSG-Glo assays. The fact that the ARE GeneBLAzer assay may also be activated by non-Nrf2 mechanisms may help to explain why such a low fraction of the observed effect can be typically explained by detected chemicals.

In contrast to the single chemicals, the majority of the studied surface water samples induced ROS formation and/or altered cellular glutathione concentrations. Reduced GSH can be oxidised to GSSG in the presence of ROS. GSSG can be converted back to GSH by glutathione reductase, but extensive oxidative stress impedes this process, leading to the accumulation of GSSG. Consequently, reduced GSH/GSSG ratio and increased GSSG formation are indicators of ROS. Twelve of the JDS samples reduced the GSH/GSSG ratio, with 11 of these 12 samples also inducing ROS formation in the DCFH-DA assay. Sample JDS 32 was one of the more active samples in the ARE GeneBLAzer assay and decreased the GSH/GSSG ratio at an REF of 41, but
did not induce ROS formation up to an REF of 80. The concentration of antioxidant genistein in JDS 32 was 166 pM, which was 2.5 to 7 times higher than any of the other samples.\(^\text{10}\) Ma et al.\(^\text{41}\) demonstrated that genistein could suppress ROS formation by β-amyloid peptides in PC12 cells, thus it is possible that the elevated genistein concentration in JDS 32 reduced ROS formation. Two samples, JDS 57 and JDS 60, increased ROS formation, but did not significantly reduce the GSH/GSSG ratio. As can be seen in Figure 1, the ROS formation EC\(_{\text{IR1.5,ROS}}\) value from the DCFH-DA assay was higher than the EC\(_{\text{IR1.5,ox stress}}\) value from the ARE GeneBLAzer assay for these two samples. The GSH/GSSH-Glo assay was run at the ARE GeneBLAzer EC\(_{\text{IR1.5,ox stress}}\) value, which explains why ROS formation did not translate into a reduced GSH/GSSG ratio.

JDS 29 and 35 did not significantly increase the GSSG concentration, but decreased the GSH/GSSG ratio and induced ROS formation in the DCFH-DA assay. Both samples decreased the reduced GSH concentration in the cells and this may indicate the presence of soft electrophiles, which can deplete GSH.\(^\text{42}\) However, this cannot be confirmed without measuring the formation of glutathione conjugates.

JDS 41 had the strongest response in the ARE GeneBLAzer assay, but did not increase ROS formation in the DCFH-DA assay or GSSG formation, or decrease the GSH/GSSG ratio. This suggests that the observed effect was not due to ROS formation. The reduced GSH concentration after exposure to JDS 41 was significantly lower than the solvent control, suggesting the presence of electrophilic chemicals. Unlike the other JDS samples, the window between induction and cytotoxicity in the ARE GeneBLAzer assay was small for JDS 41, with an EC\(_{\text{IR1.5,ox stress}}\) of 15.5 and a concentration causing 10% reduction in cell viability (EC\(_{\text{10}}\)) of 26.8. Therefore, it is possible that the observed decrease in reduced GSH is due to non-specific effects. Similarly, JDS 59 also had a strong response in the ARE GeneBLAzer assay, but did not have any significant effect in either the DCFH-DA or GSH/GSSG-Glo assays. Both JDS 41 and 59 were among the most chemically polluted sites in the Joint Danube Survey, with 61 and 64 chemicals detected, respectively, and a sum chemical concentration of 18.0 and 16.4 nM, respectively.\(^\text{10}\) The detected chemicals included pharmaceuticals, pesticides, food additives and industrial chemicals. There does not appear to be any correlation between the detected chemical concentration and ROS formation or cellular glutathione concentrations (data not shown).

Antioxidants, including the tested genistein but also other polyphenols, phenolic acids and organosulfur compounds, can induce the Nrf2 response,\(^\text{43}\) in addition to harmful ROS and electrophiles. A range of antioxidants, including isothiocyanates, flavones and polyphenols, are active in the ARE GeneBLAzer assay.\(^\text{44}\) Antioxidants can activate the Nrf2-ARE pathway due to their pro-oxidant or electrophilic properties.\(^\text{45}\) For example, the isothiocyanate sulforaphane can act as a pro-oxidant,\(^\text{46}\) flavonoids can also act as pro-oxidants after donating electrons during
antioxidant action\textsuperscript{47} and the polyphenol curcumin contains electrophilic $\alpha,\beta$-unsaturated carbonyl groups.\textsuperscript{48} By activating Nrf2, these compounds can induce protective antioxidant enzymes and increase the levels of nucleophiles, such as GSH, which helps to enhance the adaptive response of cells to further damage.\textsuperscript{45} It is possible that the JDS samples could contain other chemoprotective agents in addition to genistein. Further, mass spectrometry characterisation of dissolved organic matter (DOM) from the Danube River revealed that around 20% of the assigned formula belonged to the polyphenol compound class.\textsuperscript{49} Therefore, any co-extracted polyphenols may potentially have an effect in the assay. While the amount of DOM co-extracted by LVSPE is unknown, between 40 to 70% can be co-extracted using conventional solid phase extraction materials, such as Oasis HLB.\textsuperscript{50} As the water samples were enriched 50,000 times by LVSPE, even low organic matter extraction efficiency could still potentially lead to significant polyphenol concentrations in the assays.

**Conclusions**

Assays indicative of the oxidative stress response are increasingly being applied to both single chemicals and environmental samples \textsuperscript{10,11,51}, but the mechanisms by which the samples are activing ARE remains unclear. This is important as both oxidants as well as antioxidants may activate the Nrf2-ARE pathway. In this study, we aimed to explore the oxidative stress response further by assessing ROS formation and changes in glutathione in hepatic human cells exposed to both environmental samples and single chemicals known to be active in the ARE GeneBLAzer assay. None of the five studied chemicals increased ROS formation, reduced the GSH/GSSG ratio or depleted GSH, with the activity of genistein in the ARE GeneBLAzer assay likely to be due its antioxidant properties. In contrast, many of the environmental samples induced ROS formation in the DCFH-DA assay and/or altered the GSH/GSSG ratio in HepG2 cells, suggesting that formation of ROS is a likely mechanism. As the ARE GeneBLAzer may also respond to chemicals that can activate ARE by Nrf2-independent mechanisms, as well as antioxidants, more work is required to characterise active chemicals in this assay. As such, the use of oxidative stress response assays, such as the ARE GeneBLAzer, are not recommended as a standalone technique for environmental monitoring. However, as the oxidative stress response is integrative over many molecular initiating events, such assays are suitable for use as sum chemical parameters and can be included as part of a bioassay test battery covering different modes of action, including assays indicative of receptor-mediated effects.
Acknowledgements

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33. US EPA, Interactive Chemical Safety for Sustainability (iCSS) Dashboard v2,


36. Promega Corporation, GSH/GSSG-Glo™ Assay Technical Manual,


Table 1: ROS formation EC<sub>IR1.5</sub>,ROS and oxidative stress response EC<sub>IR1.5,ox stress</sub> values in units of relative enrichment factor, with the GSH/GSSG ratio for the studied environmental samples and positive and negative reference compounds (SD: standard deviation).

<table>
<thead>
<tr>
<th>Sample ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ROS Formation (EC&lt;sub&gt;IR1.5,ROS&lt;/sub&gt;) ± SD</th>
<th>GSH/GSSG ratio ± SD (measured at EC&lt;sub&gt;IR1.5,ox stress&lt;/sub&gt;)</th>
<th>Oxidative Stress Response (EC&lt;sub&gt;IR1.5,ox stress&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt; ± SD</th>
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<tbody>
<tr>
<td>JDS 8</td>
<td>88.3 ± 28.8</td>
<td>7.36 ± 1.15*</td>
<td>78.7 ± 3.82</td>
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<tr>
<td>JDS 22</td>
<td>45.5 ± 2.96</td>
<td>10.4 ± 1.49*</td>
<td>88.8 ± 6.06</td>
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<td>JDS 27</td>
<td>39.0 ± 3.89</td>
<td>10.6 ± 1.17*</td>
<td>68.1 ± 4.97</td>
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<td>JDS 29</td>
<td>45.5 ± 4.52</td>
<td>11.4 ± 1.99*</td>
<td>71.9 ± 11.1</td>
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<td>JDS 30</td>
<td>70.9 ± 8.74</td>
<td>8.79 ± 1.63*</td>
<td>119 ± 7.14</td>
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<tr>
<td>JDS 32</td>
<td>&gt;80</td>
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<td>41.2 ± 4.62</td>
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<td>JDS 33</td>
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<td>6.26 ± 1.16*</td>
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<td>39.5 ± 6.33</td>
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<td>JDS 37</td>
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<td>92.9 ± 19.6</td>
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<td>15.5 ± 1.39</td>
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<td>26.8 ± 4.01</td>
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<td>47.0 ± 6.66</td>
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<td>15.9 ± 3.42</td>
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<td>JDS 57</td>
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<td>15.7 ± 2.08</td>
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<td>16.7 ± 2.43</td>
<td>30.1 ± 1.25</td>
</tr>
<tr>
<td>JDS 65</td>
<td>&gt;45</td>
<td>13.3 ± 2.87</td>
<td>37.4 ± 1.37</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(5.62±0.66)×10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Menadione</td>
<td>-</td>
<td>1.33 ± 0.51</td>
<td>-</td>
</tr>
<tr>
<td>Methanol</td>
<td>-</td>
<td>17.1 ± 3.37</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Description of sampling sites and site number published in Neale et al. 10

<sup>b</sup>ARE GeneBLAzer assay, data previously published in Neale et al. 10

<sup>c</sup>EC<sub>IR1.5,ROS</sub> for H<sub>2</sub>O<sub>2</sub> in molar units

<sup>*</sup>Significantly different from methanol control <0.01 (Unpaired t-test)
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Figure 1: ROS formation EC_{IR1.5,ROS} values for the DCFH-DA assay compared with oxidative stress response EC_{IR1.5,ox stress} values for the ARE GeneBLAzer assay from Neale et al. \(^{10}\) in units of relative enrichment factor (REF). Numbers indicate the JDS site numbers\(^{10}\) and error bars indicate standard deviation.

Figure 2: Reduced GSH, GSSG and GSH/GSSG ratio for the JDS samples measured at EC_{IR1.5,ox stress} (REF) with negative control (NC) methanol and positive control (PC) menadione. Error bars indicate standard deviation and description of JDS site number is provided in Neale et al. \(^{10}\)

Figure 3: Summary of JDS samples that decreased the reduced GSH concentration, decreased the GSH/GSSG ratio and/or increased the GSSG concentration in HepG2 cells. Numbers indicate the JDS site numbers,\(^{10}\) with numbers in bold indicating samples that increased GSSG concentration, but did not induce ROS formation in the DCFH-DA assay.

Figure 4: Reduced GSH, GSSG and GSH/GSSG ratio for the studied chemicals at their US EPA ToxCast EC_{IR1.5,ox stress} value (M) (provided in Figure) with negative control (NC) methanol and positive control (PC) menadione. Error bars indicate standard deviation.
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