Exploring the oxidative stress response mechanism triggered by environmental water samples

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36 **Abstract**

37 Environmental waters can contain a wide range of micropollutants. Bioanalytical test batteries using
38 assays indicative of different stages of cellular toxicity pathways, such as adaptive stress responses,
39 have been applied to a range of water samples. Oxidative stress response assays have proven to be
40 sensitive tools, but the mechanism by which water samples are inducing the oxidative stress
41 response remains unclear because both electrophiles and reactive oxygen species (ROS) may
42 activate the Nrf2-antioxidant response element (ARE) pathway. The current study aimed to explore
43 the underlying mechanisms of the oxidative stress response triggered by exposure to surface water
44 extracts previously shown to be active in the ARE GeneBLAzer oxidative stress response assay.
45 ROS formation and changes in glutathione (GSH) concentration were assessed in human liver cells
46 exposed to water extracts from a large river in addition to individual chemicals that were detected in
47 these water extracts and reported to be active in the ARE GeneBLAzer assay in a previous study.
48 Many of the surface water samples induced ROS formation and decreased the GSH to glutathione
49 disulfide (GSSG) ratio, suggesting that the formation of ROS is an important mechanism. However,
50 some of the most responsive samples in the ARE GeneBLAzer assay, as well as the individual
51 chemicals, did not have a significant effect on either ROS formation or the GSH/GSSG ratio,
52 suggesting a different mechanism. Antioxidants can also induce the Nrf2-ARE pathway and the
53 ARE GeneBLAzer assay may also detect compounds that activate ARE by Nrf2-independent
54 mechanisms, thus further research is required to characterise active chemicals in oxidative stress
55 response assays. However, these tests are still useful for quantifying the integrated cellular response
56 to multiple molecular initiating events and can be used complementary to assays indicative of
57 specific effects, such as receptor-mediated assays.

58 **Introduction**

59 The aquatic environment can contain a diverse range of organic micropollutants and exposure to
60 these contaminants can potentially have implications for both ecological and human health.^{1,2} Water
61 quality monitoring typically utilises targeted chemical analysis. However, because of the wide range
62 of contaminants and their transformation products in the environment, targeted chemical analysis
63 can only provide partial information. As a result, *in vitro* bioanalytical tools are applied alongside to
64 provide information about the effect of all chemicals in a sample.³ A recent study that applied over
65 100 *in vitro* bioassays to a range of water samples found that oxidative stress response assays were
66 among the most responsive assays tested.⁴ The oxidative stress response is an important cellular
67 adaptive stress response pathway and is typically activated at lower chemical concentrations than
68 those that cause cell death.⁵ *In vitro* bioassays indicative of the oxidative stress response, such as
69 AREc32 and ARE GeneBLAzer, have been applied to individual chemicals,⁶⁻⁸ chemical mixtures⁹
70 and environmental samples, including drinking water, surface water and wastewater.¹⁰⁻¹² The
71 oxidative stress response assays have proven to be sensitive tools, but mixture toxicity modelling
72 studies have shown that detected chemicals can only explain a small fraction (<2%) of the observed
73 oxidative stress response in water samples.^{9,13}

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

87 Not all electrophiles will react with the sulfhydryl group. Electrophiles can be classified as
88 hard or soft based on their molecular properties, with soft electrophiles typically large, highly
89 polarizable molecules, while hard electrophiles are smaller molecules with low polarizability.²²
90 Biological nucleophiles can also be classified as hard, such as DNA, or soft, such as the sulfhydryl
91 group of cysteine residues, based on their polarizability and electrophiles prefer to react with
92 nucleophiles of a similar softness or hardness.^{23,24} Thus soft electrophiles, which include many

disinfection by-products,⁸ the biocide Sea-Nine²⁵ and the industrial compound ethyl acrylate,²³ 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.²⁶⁻²⁸ 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.²⁹ 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.³⁰

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.¹⁰ 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'-Dichlorofluorescein 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).

128 Water samples

129 Large volume solid phase extraction (LVSPE) water samples from the 2013 Joint Danube Survey
 130 (JDS) were applied in this study. The samples have been extensively characterised by both chemical
 131 analysis and bioanalysis, with the majority of samples found to induce oxidative stress in the ARE
 132 GeneBLAzer assay.¹⁰ Only samples that were active in the ARE GeneBLAzer assay were included
 133 in this study. Information about sample enrichment and extraction, as well as sampling locations,
 134 can be found in the Supporting Information of Neale *et al.*¹⁰, with the JDS site numbers referring to
 135 the location of the sampling sites along the Danube River. Relative enrichment factors (REF) were
 136 used as concentration measures for the water samples and provide an indication of the sample
 137 enrichment or dilution required to see an effect in the assay.³¹ The REF (Equation 1) was calculated
 138 using the sample enrichment factor after LVSPE, which was based on the total volume of water
 139 sampled and the final volume of the sample extract (Equation 2), and the assay dilution factor
 140 (Equation 3).³²

$$\text{REF} = \text{dilution factor}_{\text{assay}} \cdot \text{enrichment factor}_{\text{LVSPE}} \quad (1)$$

$$\text{enrichment factor}_{\text{LVSPE}} = \frac{\text{total volume of water}}{\text{volume of extract}} \quad (2)$$

$$\text{dilution factor}_{\text{assay}} = \frac{\text{volume of extract added to assay}}{\text{total volume of assay}} \quad (3)$$

148 The sample REF for the GSH/GSSG-Glo assay was the effect concentration causing an
 149 induction ratio of 1.5 ($\text{EC}_{\text{IR1.5,ox stress}}$) in the ARE GeneBLAzer assay,¹⁰ while a three-point dilution
 150 series starting at double the $\text{EC}_{\text{IR1.5,ox stress}}$ was used for samples in the DCFH-DA assay. $\text{EC}_{\text{IR1.5,ox}}$
 151 stress indicates a 50% increase in effect compared to the negative control and is close to the assay
 152 limit of detection.¹¹ Cell viability was assessed in parallel to induction using resazurin in Neale *et*
 153 *al.*¹⁰ and cytotoxic concentrations were excluded from the DCFH-DA and GSH/GSSG assays. The
 154 five chemicals were analysed in the DCFH-DA and GSH/GSSG-Glo assays at concentrations in the
 155 range of their ARE GeneBLAzer $\text{EC}_{\text{IR1.5,ox stress}}$ values (Table S1). The $\text{EC}_{\text{IR1.5,ox stress}}$ values were
 156 derived using raw emission data from the US EPA ToxCast database,³³ with further information
 157 about the data evaluation method found in Neale *et al.*¹⁰

158 *Cell culture*

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

163

164 *DCFH-DA assay*

165 Intracellular ROS formation was assessed using the DCFH-DA assay, where DCFH-DA is taken up
166 by intact cells and enzymatically hydrolysed to non-fluorescent DCFH by cellular esterases.³⁴
167 DCFH is then oxidised to fluorescent DCF in the presence of ROS. The DCFH-DA assay was
168 conducted based on the protocol outlined in Wang and Joseph³⁵, with some modifications. Briefly,
169 100 µL of HepG2 cells in DMEM with GlutaMAX™ were seeded at 2×10⁵ cells/mL in 96 well
170 black clear bottom plates and incubated for 24 h at 37°C, 5% CO₂. After 24 h the medium was
171 removed and the cells were washed twice with phosphate-buffered saline (PBS), then 100 µL of
172 phenol red free DMEM containing 100 µM DCFH-DA was added to the cells. After incubating at
173 37°C in the dark for 30 min, the medium was removed and the cells were again washed twice with
174 PBS, then 100 µL of water extract or test chemical serially diluted in PBS was added. All samples
175 were prepared in methanol, with the solvent concentration in the assay not exceeding 1% to prevent
176 any solvent effects. H₂O₂ served as the positive control, while PBS only served as the negative
177 control. Fluorescence at 485 and 520 nm was measured using a Fluostar Omega microplate reader
178 (BMG Labtech, Ortenberg, Germany), with readings taken immediately after sample addition (t=0)
179 and at 2 h (t=2). Each sample was run in triplicate per experiment, with each experiment conducted
180 at least twice. Percent fluorescence increase at 2 h was calculated for both the samples and PBS
181 controls using Equation 4, where RFU refers to relative fluorescence units. The fluorescence
182 induction ratio was calculated using Equation 5. Linear concentration-effect curves with an
183 intercept of zero and a fitted slope were used to determine the effect concentration causing a ROS
184 formation induction ratio of 1.5 (EC_{IR1.5,ROS}) (Equation 6).⁴

185

$$\%_{\text{increase } t=2} = \frac{\text{RFU}_{\text{sample or PBS } t=2} - \text{RFU}_{\text{PBS } t=0}}{\text{RFU}_{\text{PBS } t=0}} \quad (4)$$

186

187

$$\text{Induction ratio (IR)} = \frac{\%_{\text{increase sample } t=2}}{\%_{\text{increase PBS } t=2}} \quad (5)$$

188

189

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

190

(6)

191

192 *GSH/GSSG-Glo Assay*

193 Reduced GSH, GSSG and the GSH/GSSG ratio were determined using the GSH/GSSG-Glo assay,
 194 which is based on the conversion of a luciferin derivative GSH probe to luciferin by GST.³⁶ The
 195 GSH/GSSG-Glo assay was conducted according to the Promega technical manual³⁶, with some
 196 modifications. Briefly, the samples were diluted in Krebs-Henseleit buffer (Section S1 and Table S2
 197 of the Supplementary Information) in a 96 well plate, then 4 µL of pre-diluted sample was added to
 198 a white low volume 384 well plate. Each sample had 4 replicates for both total glutathione and
 199 GSSG analysis. The final sample concentration in the assay was the $EC_{IR1.5,ox\ stress}$ values in units of
 200 REF or M (Tables 1 and S1). HepG2 cells were washed twice and resuspended in Krebs-Henseleit
 201 buffer at a concentration of 2.5×10^5 cells/mL, with 4 µL added to each well, giving a concentration
 202 of 1,000 cells/well. The cells were exposed to the samples for 1 h at 37°C, then 2 µL of either Total
 203 Glutathione Lysis Reagent (Promega) for total glutathione analysis or Oxidised Glutathione Lysis
 204 Reagent (Promega) for GSSG analysis was added for cell lysis and the samples were shaken for 5
 205 min at 500 rpm. After shaking, 2 µL of Luciferin Generation Reagent (Promega) was added to each
 206 well and the samples were incubated for 30 min at room temperature. Finally, 8 µL of Luciferin
 207 Detection Reagent (Promega) was added to each well and allowed to equilibrate for 15 min at room
 208 temperature, and then luminescence in relative light units (RLU) was measured using a Fluostar
 209 Omega microplate reader. Menadione served as the positive control and methanol was the solvent
 210 control, while cell-free controls were also included. A GSH standard curve from 0.5 to 8 µM in the
 211 absence of cells was also included in each experiment to convert RLU to total GSH concentration.
 212 GSSG concentration was calculated from the standard curve with the GSH concentration divided by
 213 two as one mole of GSSG is equivalent to two moles of GSH in the GSH/GSSG assay³⁶. The cell-
 214 free RLU was subtracted from the sample RLU. The GSH/GSSG ratio was calculated for each
 215 sample using Equation 7, while reduced GSH was calculated by subtracting GSSG from total GSH.
 216 Statistically significant changes in reduced GSH, GSSG and GSH/GSSG ratio compared to the
 217 solvent control were determined using a two-tailed t-test (p-value < 0.01).

218

$$\text{GSH/GSSG ratio} = \frac{\text{Total GSH } (\mu\text{M}) - (2 \cdot \text{GSSG } (\mu\text{M}))}{\text{GSSG } (\mu\text{M})}$$

219

(7)

220 **Results**

221 *ROS formation*

222 Nineteen JDS samples were tested in the DCFH-DA assay, with the concentration-effect curves
223 shown in Figure S1 of the Supplementary Information. The majority of the surface water samples
224 induced ROS formation in the studied concentration range, with the exception of JDS 32, 41, 53,
225 59, 63 and 65. $EC_{IR1.5,ROS}$ values were calculated for the active samples and compared with the ARE
226 GeneBLAzer $EC_{IR1.5,ox\ stress}$ values previously published in Neale *et al.*¹⁰ (Figure 1). Most of the
227 active samples induced ROS at lower or similar REFs as induced the oxidative stress response in
228 the ARE GeneBLAzer assay, though JDS 57 and 60 only induced ROS at concentrations higher
229 than the respective ARE GeneBLAzer $EC_{IR1.5,ox\ stress}$ values (Table 1). Five chemicals detected in
230 these water samples and reported to be active in the ARE GeneBLAzer assay were also tested in the
231 DCFH-DA assay at concentrations in the range of their ARE GeneBLAzer $EC_{IR1.5,ox\ stress}$ values
232 (Table S1). None of the chemicals increased ROS formation in the studied concentration range
233 (Figure S2).

235 *Changes in glutathione concentration*

236 Reduced GSH and GSSG concentrations, as well as the GSH/GSSG ratio, were determined for the
237 JDS samples using the GSH/GSSG-Glo assay (Figure 2), with all samples tested at the REF
238 inducing $EC_{IR1.5,ox\ stress}$ in the ARE GeneBLAzer assay. Compared to the methanol control, 13 JDS
239 samples (JDS 8, 22, 27, 29, 30, 32, 33, 35, 36, 37, 39, 41 and 44) significantly decreased the
240 reduced GSH concentration in the cells, while 15 samples (JDS 8, 22, 27, 30, 32, 33, 36, 37, 39, 44,
241 53, 57, 60, 63 and 65) increased the GSSG concentration. Twelve samples, JDS 8, 22, 27, 29, 30,
242 32, 33, 35, 36, 37, 39 and 44, also significantly decreased the GSH/GSSG ratio. The active samples
243 are summarised in Figure 3.

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

248 **Discussion**

249 Environmental waters, such as surface waters, can contain a wide range of compounds and their
250 transformation products. Of the 7,522 chemicals analysed in the ARE GeneBLAzer assay as part of
251 the US EPA ToxCast program, 23% were reported to be active in the assay.³³ Consequently, many
252 of the chemicals present in the JDS samples may contribute to the observed oxidative stress
253 response. Of the 92 chemicals detected in the studied JDS samples, 15 were reported to be active in
254 the ARE GeneBLAzer assay, according to the most recent version of the US EPA ToxCast

255 Database.³³ The five chemicals selected for further study were prioritised based their frequency of
256 occurrence in the JDS samples (present in 42 to 100% of the studied samples), detected
257 concentration (e.g. caffeine found at concentrations up to 1029 pM) and reported effect in the US
258 EPA ToxCast database. With the exception of metolachlor, which increased the reduced GSH
259 concentration in HepG2 cells, none of the five studied chemicals were responsive in the DCFH-DA
260 or GSH/GSSG-Glo assays. Wu *et al.*³⁷ reported increased ROS formation in HepG2 cells in the
261 presence of diclofenac, but only at higher concentrations than tested in the current study. Increased
262 GSH concentration in the presence of metolachlor was also previously observed by Dierickx³⁸ in
263 HepG2 cells, with the increased GSH concentration attributed to cellular defence mechanisms. In
264 contrast, the phytoestrogen genistein is an antioxidant and was reported to increase Nrf2 expression
265 in Caco-2 cells via protein kinase pathways, leading to activation of the ARE.³⁹ The mechanisms by
266 which antioxidants can induce the Nrf2-ARE pathway are discussed in further detail below.

267 The ToxCast database also includes the Attagene Nrf2/ARE-cisFACTORIAL assay using
268 HepG2 cells, but only 2,4-dinitrophenol and metolachlor were reported as active.³³ Further,
269 caffeine, genistein and metolachlor were reported to be inactive in the Aprelica 24 h HepG2
270 oxidative stress assay (2,4-dinitrophenol and diclofenac were not tested).³³ The ARE GeneBLAzer
271 assay was constructed using a human NAD(P)H:quinone oxidoreductase 1 (NQO1) gene, which has
272 the potential to bind other transcription factors, such as c-Jun and MafK, in addition to Nrf2, with
273 some likely to be positive factors and activate ARE, while others may downregulate expression.⁴⁰
274 Further, the ARE GeneBLAzer assay may be susceptible to false positives due to the assay
275 specifications, such as promoter construction and ARE orientation, which enables ARE
276 GeneBLAzer to potentially detect compounds that activate ARE through non-specific mechanisms,
277 in addition to Nrf2 dependent mechanisms.⁷ This may explain why some of the studied chemicals
278 known to be active in the ARE GeneBLAzer assay did not have any effect in the DCFH-DA and
279 GSH/GSSG-Glo assays. The fact that the ARE GeneBLAzer assay may also be activated by non-
280 Nrf2 mechanisms may help to explain why such a low fraction of the observed effect can be
281 typically explained by detected chemicals.^{10,13}

282 In contrast to the single chemicals, the majority of the studied surface water samples
283 induced ROS formation and/or altered cellular glutathione concentrations. Reduced GSH can be
284 oxidised to GSSG in the presence of ROS. GSSG can be converted back to GSH by glutathione
285 reductase, but extensive oxidative stress impedes this process, leading to the accumulation of
286 GSSG.³⁰ Consequently, reduced GSH/GSSG ratio and increased GSSG formation are indicators of
287 ROS. Twelve of the JDS samples reduced the GSH/GSSG ratio, with 11 of these 12 samples also
288 inducing ROS formation in the DCFH-DA assay. Sample JDS 32 was one of the more active
289 samples in the ARE GeneBLAzer assay and decreased the GSH/GSSG ratio at an REF of 41, but

290 did not induce ROS formation up to an REF of 80. The concentration of antioxidant genistein in
291 JDS 32 was 166 pM, which was 2.5 to 7 times higher than any of the other samples.¹⁰ Ma *et al.*⁴¹
292 demonstrated that genistein could suppress ROS formation by β -amyloid peptides in PC12 cells,
293 thus it is possible that the elevated genistein concentration in JDS 32 reduced ROS formation. Two
294 samples, JDS 57 and JDS 60, increased ROS formation, but did not significantly reduce the
295 GSH/GSSG ratio. As can be seen in Figure 1, the ROS formation $EC_{IR1.5,ROS}$ value from the DCFH-
296 DA assay was higher than the $EC_{IR1.5,ox\ stress}$ value from the ARE GeneBLAzer assay for these two
297 samples. The GSH/GSSH-Glo assay was run at the ARE GeneBLAzer $EC_{IR1.5,ox\ stress}$ value, which
298 explains why ROS formation did not translate into a reduced GSH/GSSG ratio.

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

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

319 Antioxidants, including the tested genistein but also other polyphenols, phenolic acids and
320 organosulfur compounds, can induce the Nrf2 response,⁴³ in addition to harmful ROS and
321 electrophiles. A range of antioxidants, including isothiocyanates, flavones and polyphenols, are
322 active in the ARE GeneBLAzer assay.⁴⁴ Antioxidants can activate the Nrf2-ARE pathway due to
323 their pro-oxidant or electrophilic properties.⁴⁵ For example, the isothiocyanate sulforaphane can act
324 as a pro-oxidant,⁴⁶ flavonoids can also act as pro-oxidants after donating electrons during

antioxidant action⁴⁷ and the polyphenol curcumin contains electrophilic α,β -unsaturated carbonyl groups.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.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.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.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^{10,11,51}, but the mechanisms by which the samples are activating 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.

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Table 1: ROS formation $EC_{IR1.5,ROS}$ and oxidative stress response $EC_{IR1.5,ox\ stress}$ 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).

Sample ID ^a	ROS Formation ($EC_{IR1.5,ROS}$) $\pm$ SD	GSH/GSSG ratio $\pm$ SD (measured at $EC_{IR1.5,ox\ stress}$)	Oxidative Stress Response ($EC_{IR1.5,ox\ stress}$) ^b $\pm$ SD
JDS 8	88.3 $\pm$ 28.8	7.36 $\pm$ 1.15*	78.7 $\pm$ 3.82
JDS 22	45.5 $\pm$ 2.96	10.4 $\pm$ 1.49*	88.8 $\pm$ 6.06
JDS 27	39.0 $\pm$ 3.89	10.6 $\pm$ 1.17*	68.1 $\pm$ 4.97
JDS 29	45.5 $\pm$ 4.52	11.4 $\pm$ 1.99*	71.9 $\pm$ 11.1
JDS 30	70.9 $\pm$ 8.74	8.79 $\pm$ 1.63*	119 $\pm$ 7.14
JDS 32	>80	8.01 $\pm$ 1.37*	41.2 $\pm$ 4.62
JDS 33	55.1 $\pm$ 8.75	6.26 $\pm$ 1.16*	67.5 $\pm$ 6.58
JDS 35	13.8 $\pm$ 1.61	11.5 $\pm$ 1.23*	25.5 $\pm$ 1.27
JDS 36	39.5 $\pm$ 6.33	10.9 $\pm$ 1.97*	68.0 $\pm$ 3.43
JDS 37	93.6 $\pm$ 14.7	8.22 $\pm$ 2.79*	87.5 $\pm$ 1.86
JDS 39	37.8 $\pm$ 3.87	9.97 $\pm$ 1.22*	92.9 $\pm$ 19.6
JDS 41	>25	18.6 $\pm$ 4.41	15.5 $\pm$ 1.39
JDS 44	26.8 $\pm$ 4.01	8.00 $\pm$ 3.75*	47.0 $\pm$ 6.66
JDS 53	>80	15.9 $\pm$ 3.42	42.9 $\pm$ 3.83
JDS 57	79.9 $\pm$ 19.4	15.7 $\pm$ 2.08	60.1 $\pm$ 2.34
JDS 59	>80	13.53 $\pm$ 2.57	41.8 $\pm$ 1.90
JDS 60	64.5 $\pm$ 12.6	14.2 $\pm$ 2.08	44.4 $\pm$ 2.85
JDS 63	>45	16.7 $\pm$ 2.43	30.1 $\pm$ 1.25
JDS 65	>45	13.3 $\pm$ 2.87	37.4 $\pm$ 1.37
H ₂ O ₂ ^c	(5.62 $\pm$ 0.66) $\times 10^{-5}$	-	-
Menadione	-	1.33 $\pm$ 0.51	-
Methanol	-	17.1 $\pm$ 3.37	-

^aDescription of sampling sites and site number published in Neale *et al.*¹⁰

^bARE GeneBLAzer assay, data previously published in Neale *et al.*¹⁰

^c $EC_{IR1.5,ROS}$ for H₂O₂ in molar units

*Significantly different from methanol control <0.01 (Unpaired t-test)

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474 **Figure 1:** ROS formation $EC_{IR1.5,ROS}$ values for the DCFH-DA assay compared with oxidative
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476 relative enrichment factor (REF). Numbers indicate the JDS site numbers¹⁰ and error bars indicate
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478

479 **Figure 2:** Reduced GSH, GSSG and GSH/GSSG ratio for the JDS samples measured at $EC_{IR1.5,ox}$
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481 indicate standard deviation and description of JDS site number is provided in Neale *et al.*¹⁰

482

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488 **Figure 4:** Reduced GSH, GSSG and GSH/GSSG ratio for the studied chemicals at their US EPA
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