What is driving the NF- κ B response in environmental water extracts?

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1 **Abstract**

2 *In vitro* bioassays are increasingly applied for water quality monitoring, with assays indicative of
3 adaptive stress responses commonly included in test batteries. The NF- κ B assay is responsive to
4 surface water and wastewater extracts, but the causative compounds are unknown and
5 micropollutants typically found in water do not activate the NF- κ B assay. The current study aimed
6 to investigate if co-extracted organic matter and/or endotoxins could cause the NF- κ B response in
7 surface water extracts. The effect of model bacterial lipopolysaccharides (LPS) and dissolved
8 organic carbon (DOC) was evaluated in the NF- κ B assay both before and after solid-phase
9 extraction (SPE), with 7% effect recovery for LPS and between 7 to 52% effect recovery for DOC
10 observed. The NF- κ B response, endotoxin activity, micropollutant concentration and total organic
11 carbon concentration was measured in four surface water extracts. All water extracts showed a
12 response in the NF- κ B assay, but the detected micropollutants could not explain the effect.
13 Comparison of predicted bioanalytical equivalent concentrations based on micropollutant, DOC and
14 endotoxin concentrations in surface water with experimental bioanalytical equivalent concentrations
15 suggest that co-extracted endotoxins are the most important drivers of the observed effect, with
16 DOC only having a minor contribution. While *in vitro* bioassays typically detect mixtures of
17 organic micropollutants, the current study shows that the NF- κ B assay can integrate the effects of
18 co-extracted endotoxins. Given that endotoxins can pose a risk for human health, the NF- κ B assay
19 is a valuable inclusion in bioanalytical test batteries used for water quality monitoring.

20

21 **Keywords:** bioassay; dissolved organic carbon; endotoxin; lipopolysaccharide; solid-phase
22 extraction; surface water

23

24 **Abbreviations:** BEQ: bioanalytical equivalent concentration; DOC: dissolved organic carbon; EC:
25 effect concentration; EU: endotoxin units; FBS: fetal bovine serum; HA: humic acid; HILIC:
26 hydrophilic interaction chromatography; IR: induction ratio; LAL: *Limulus amoebocyte* lysate; LC-

27 MS/MS: liquid chromatography tandem mass spectrometry; LPS: lipopolysaccharides; LVSPE:
28 large volume solid-phase extraction; NATA: National Association of Testing Authorities; NOM:
29 natural organic matter; REF: relative enrichment factor; REP: relative effect potency; ROS: reactive
30 oxygen species; SPE: solid-phase extraction; TNF α : Tumor Necrosis Factor alpha

31

32 **1. Introduction**

33 *In vitro* bioassays are increasingly applied for water quality monitoring to detect the effects of
34 complex chemical mixtures (Schroeder et al., 2016; Tousova et al., 2017). Bioassay test batteries
35 covering different stages of cellular toxicity pathways such as induction of xenobiotic metabolism
36 and receptor-mediated effects, as well as apical effects in whole organisms, have been
37 recommended to ensure that a range of possible effects in water are detected (Neale et al., 2017a).
38 This also includes assays indicative of adaptive stress response pathways, such as the oxidative
39 stress response and NF- κ B response, which help to restore the cell to homeostasis after damage
40 from stressors (Simmons et al., 2009). NF- κ B transcription factors are important for immune and
41 inflammation responses, as well as for the growth and development of the cell (Oeckinghaus and
42 Ghosh, 2009). NF- κ B is complexed with inhibitory I κ B proteins in the cytoplasm of most cells and
43 is inactive (Baeuerle and Baltimore, 1988). After exposure to stimuli, I κ B can be degraded by
44 phosphorylation by the I κ B kinase pathway, which releases NF- κ B from the complex and allows it
45 activate target genes in the nucleus (Gilmore, 2006). The types of stimuli that can destabilise the
46 I κ B-NF- κ B complex are wide ranging and include bacterial products, metals, carcinogens and
47 compounds in cigarette smoke (Ahn and Aggarwal, 2005).

48 Assays indicative of the NF- κ B response have been recently applied to drinking water,
49 surface water and wastewater samples after enrichment (König et al., 2017; Neale et al., 2017b;
50 Hebert et al., 2018; Nivala et al., 2018), with strong responses observed in wastewater and surface
51 water (Figure 1). However, it is still unclear which environmental chemicals are inducing an effect
52 in the assay. The US EPA ToxCast database screened over 7500 chemicals in the NF- κ B assay,
53 with only 3.4% of the tested chemicals reported to be active (US EPA, 2015). However, most of the
54 chemicals are only active around concentrations where reduced cell viability is observed, thus this
55 activity is likely to be related to the “cytotoxicity burst” phenomenon, where reporter gene assays
56 can be activated non-specifically at or close to cytotoxic concentrations (Judson et al., 2016).
57 Further, analysis of fifty drinking water disinfection by-products found that none were active in the

58 NF- κ B assay (Stalter et al., 2016). If environmental chemicals are not activating the NF- κ B
59 response in water samples, what else could be inducing a response?

60 While bioanalysis of unenriched wastewater samples using concentrated cell medium was
61 recently demonstrated (Niss et al., 2018), sample enrichment is typically required prior to
62 bioanalysis of trace micropollutants to improve the method detection limit, particularly for less
63 polluted samples, such as surface water and drinking water. Solid-phase extraction (SPE), which
64 targets organic micropollutants and excludes inorganics including metals and salts, is a commonly
65 used enrichment method (e.g. Jugan et al., 2009; Mehinto et al., 2015; Rosenmai et al., 2018). A
66 limitation of SPE is that it may not extract all bioactive compounds, though a recent study found
67 acceptable effect recovery by large volume solid-phase extraction (LVSPE) for a number of *in vitro*
68 bioassays (Neale et al., 2018). While polymeric SPE materials, such as Oasis HLB, are often used
69 for enrichment of water samples as they are able to extract organic chemicals with a wide range of
70 physiochemical properties, they can also co-extract other matrix components in water, such as
71 dissolved organic carbon (DOC) (Neale and Escher, 2013). The possibility that co-extracted matrix
72 components in SPE extracts may be contributing to the NF- κ B response is supported by the fact that
73 no effect was observed in the NF- κ B assay for silicone rubber and Empore disk passive sampler
74 extracts from the Danube river (Novák et al., 2018), while extracts from LVSPE targeting both
75 neutral and charged chemicals also from the Danube river had a strong response in the same assay
76 (Neale et al., 2015a).

77 Bacterial lipopolysaccharides (LPS) are well-known NF- κ B activators (Sen and Baltimore,
78 1986), and endotoxins, which are naturally occurring complex LPS, are present in surface water and
79 wastewater (O'Toole et al., 2008). Studies have related inflammatory responses *in vitro* to
80 endotoxin concentrations in whole water samples (Wichmann et al., 2004; El Marghani et al.,
81 2014), but few have considered SPE extracts. Recently, Fung et al. (2017) found between 83 to
82 105% recovery of LPS in 100 μ L of ultrapure water and wastewater using microelution hydrophilic
83 interaction chromatography (HILIC) SPE with a silica-based aminopropyl sorbent. The recovery of

84 LPS reduced to 55% in 10 mL of ultrapure water. While this study used relatively small sample
85 volumes and a SPE material not commonly used for water quality monitoring, it demonstrates that
86 LPS can be co-extracted by SPE.

87 The current study aims to understand the drivers of the NF- κ B response in surface water
88 extracts. Surface waters can contain a complex mixture of micropollutants, such as pharmaceuticals,
89 personal care products and pesticides, and other matrix components, such as DOC and LPS, which
90 can be extracted by SPE to varying degrees (Figure 2). The effect of surface water extracts, as well
91 as model LPS and DOC before and after SPE, was assessed in the NF- κ B assay (Figure 2). Firstly,
92 the recovery of model LPS by different SPE sorbents previously used for water quality monitoring
93 was evaluated using the NF- κ B assay. Secondly, the effect of four surface water extracts was
94 quantified using the NF- κ B assay, with the endotoxin concentration, micropollutant concentration
95 and organic carbon properties of the surface waters characterised. Some pharmaceuticals can inhibit
96 the NF- κ B response (Khalaf et al., 2009; Miller et al., 2010), so surface waters expected to have low
97 micropollutant concentrations were selected in the current study. The effect of model DOC, humic
98 acid (HA) and natural organic matter (NOM), was also evaluated, with effect recovery by SPE
99 determined. Finally, using a mass balance approach, the contribution of micropollutants, DOC and
100 endotoxins to the NF- κ B response in surface water was estimated. The findings of the study can
101 help improve our current understanding on the suitability of the NF- κ B assay for water quality
102 monitoring.

103

104 **2. Materials and Methods**

105 *2.1. Materials*

106 All chemicals were of analytical grade. Purified lipopolysaccharide (LPS) from *Escherichia coli*
107 O111:B4 was purchased from Sigma Aldrich (Castle Hill, Australia). Suwannee River II Standard
108 HA (2S101H) and Suwannee River aquatic NOM (2R101N) were purchased from the International
109 Humic Substance Society (St Pauls, USA). Three different SPE sorbents were used in the current

110 study, Oasis HLB (500 mg sorbent) (Waters, Rydalmere, Australia), Strata-X (500 mg sorbent)
111 (Phenomenex, Lane Cove, Australia) and Supelclean Coconut Charcoal (2 g sorbent) (Sigma
112 Aldrich, Castle Hill, Australia).

113

114 2.2. LPS and DOC extraction

115 To evaluate whether common SPE sorbents can co-extract LPS a 1 µg/L LPS solution was prepared
116 in 1 L of ultrapure water. The bottles were shaken at 70 RPM for 20 min, then the pH was adjusted
117 to 3 using HCl. The three studied SPE sorbents, Oasis HLB, Strata-X and coconut charcoal, were
118 conditioned using 2 × 5 mL hexane:acetone, 2 × 5 mL methanol and 2 × 5 mL pH 3 ultrapure water,
119 then one litre of sample was extracted (and enriched) per cartridge. After drying, the cartridges were
120 eluted using 2 × 5 mL methanol and 2 × 5 mL hexane:acetone. The extracts were blown to dryness
121 under a gentle nitrogen stream and then resuspended in 500 µL methanol, giving a final enrichment
122 factor of 2000. Duplicate LPS samples were included for each SPE sorbent, while ultrapure water
123 without LPS was also enriched for each sorbent as a control. It should be noted that the SPE
124 protocol was developed specifically for enriching water samples using Oasis HLB, but was applied
125 to all three SPE sorbents for consistency. To evaluate whether model DOC had an effect after SPE,
126 solutions containing 12 mg of carbon per litre (mg_C/L) of HA or NOM were prepared in ultrapure
127 water. After adjusting the water to pH 3, the samples were enriched by Oasis HLB using the
128 protocol described above.

129

130 2.3. Surface water collection and enrichment for bioanalysis

131 Three litre surface water grab samples were collected in amber glass bottles on the 12th December
132 2017. Three of the surface water samples were collected from drinking water reservoirs (SW01,
133 SW02 and SW03) in Northern New South Wales and Southeast Queensland, Australia, with one
134 surface water sample collected from an urban creek downstream of a drinking water reservoir
135 (SW04) in Southeast Queensland (Table 1). One litre of water from each site was enriched using

136 Oasis HLB SPE cartridges on the same day as collection using the protocol described above. The
137 remaining sample volume was used for further characterisation of the surface water.

138

139 2.4. Surface water characterisation

140 The endotoxin concentration in units of endotoxin units per millilitre (EU/mL) was quantified using
141 the Pierce *Limulus ameobocyte* lysate (LAL) chromogenic endotoxin quantitation kit (Thermo
142 Fisher, Scoresby, Australia) according to the manufacturer's instructions. The samples were diluted
143 1:100, 1:200 and 1:500 in endotoxin-free water prior to analysis. Endotoxin-free controls were
144 included, as well as a four point standard curve with *E. coli* O111:B4.

145 Fifty pharmaceuticals, two biocides and two food additives were analyzed at a commercial
146 National Association of Testing Authorities (NATA) of Australia accredited laboratory using SPE
147 and liquid chromatography tandem mass spectrometry (LC-MS/MS). Some of the analyzed
148 pharmaceuticals, such as ibuprofen and sulfamethoxazole, have been shown to induce NF- κ B
149 activity in T24 cells, while oxazepam inhibited NF- κ B activity (Khalaf et al., 2009). All analyzed
150 chemicals are provided in Table S1 of the Supplementary Material.

151 To characterise the organic carbon properties, total organic carbon was measured at a NATA
152 accredited laboratory based on APHA Standard Method 5310 D (APHA, 2012), while UV-visible
153 (UV-vis) absorbance was measured at a wavelength of 254 nm.

154

155 2.5. NF- κ B assay

156 Activation of the NF- κ B response by the SPE extracts, as well as unenriched LPS and DOC, was
157 assessed using the NF- κ B-*bla* assay (Life Technologies, Mulgrave, Australia). The assay is based
158 on the human monocytic THP-1 cell line and contains a β -lactamase reporter gene that is regulated
159 by the NF- κ B response element. The assay was conducted using the protocol outlined in Neale et al.
160 (2015a). Briefly, the SPE extracts were blown down to dryness and resolubilized in RPMI 1640
161 media with 10% dialyzed fetal bovine serum (FBS), while LPS and DOC stocks were prepared

162 directly in the media. The extracts were serially diluted using a 1:2 dilution series, with 8 μ L of
163 serially diluted sample added to 32 μ L of cells with a density of 6.3×10^5 cells/mL in a black clear
164 bottom 384 well plate. The plate was incubated for 24 h at 37°C, then 8 μ L of FRET reagent with
165 resazurin for cell viability assessment was added to each well.

166 To determine if DOC was interfering with the fluorescence measurements, fluorescence was
167 measured at 460 nm (blue) and 520 nm (green) directly after the addition of the FRET reagent (0 h),
168 as well as after the standard 2 h incubation at room temperature. Resazurin fluorescence was
169 measured at 590 nm, thus DOC was unlikely to interfere with the cell viability measurements. The
170 background corrected emission ratio was calculated based on Neale et al. (2015b), with further
171 information provided in Section S1.

172 Tumor Necrosis Factor alpha (TNF α) (Life Technologies, Mulgrave, Australia) and LPS
173 from *E. coli* O111:B4 served as the positive reference compounds, while solvent controls and cell-
174 free controls were also included on each plate. All samples were run two to three times on different
175 days. The concentration-effect curves for TNF α and LPS are provided in Figure S1.

176

177 2.6. Data analysis

178 The effect in the NF- κ B assay was expressed as an induction ratio (IR) and linear concentration-
179 effect curves were used to determine the concentration causing an IR of 1.5 (EC_{IR1.5}). Further
180 information about data evaluation can be found in Escher et al. (2014) and Neale et al. (2017a). The
181 EC_{IR1.5} values were in units of μ g/L for LPS and mg_C/L for DOC, while the SPE extracts were in
182 units of relative enrichment factor (REF), which takes into account the sample enrichment factor
183 and the dilution factor in the assay (Escher and Leusch, 2012). The maximum REFs tested were
184 REF 185 for the Strata-X extracts, REF 138 for the Oasis HLB and coconut charcoal extracts, REF
185 13 for the surface water extracts and REF 185 for the DOC extracts. The EC_{IR1.5} values were
186 converted to bioanalytical equivalent concentrations (BEQ_{bio}) in units of μ g_{LPS}/L using Equation 1,
187 with LPS as the reference compound.

$$BEQ_{bio} = \frac{EC_{IR1.5} (LPS)}{EC_{IR1.5} (sample)} \quad (1)$$

Effect recovery of LPS by SPE was calculated using Equation 2 with the BEQ_{bio} value of the LPS solution extract ($BEQ_{bio, extract} (water+LPS)$) and the added LPS concentration (C_{LPS}).

$$\text{Effect recovery by SPE} = \frac{BEQ_{bio, extract} (water+LPS)}{C_{LPS}} \quad (2)$$

Effect recovery of DOC by SPE was calculated using Equation 3, with the BEQ_{bio} value of the DOC solution extract ($BEQ_{bio, extract} (water+DOC)$) and the BEQ_{bio} value of unenriched DOC ($BEQ_{bio} (DOC)$). Before calculating $BEQ_{bio} (DOC)$, the $EC_{IR1.5}$ of the unenriched DOC in units of mg_C/L was converted to the corresponding REF of a water sample with $12 mg_C/L$.

$$\text{Effect recovery by SPE} = \frac{BEQ_{bio, extract} (water+DOC)}{BEQ_{bio} (DOC)} \quad (3)$$

3. Results and Discussion

3.1. LPS is co-extracted by SPE using Oasis HLB

The recovery of *E. coli* LPS in ultrapure water by three different SPE sorbents was evaluated. Two of the sorbents, Oasis HLB and Strata-X, are polymeric sorbents, poly(divinylbenzene-co-N-vinylpyrrolidone) and poly(styrene-divinylbenzene-co-N-vinylpyrrolidone), respectively, and both have been applied for bioanalysis of environmental samples (Maletz et al., 2013; Hebert et al.,

209 2018; Leusch et al., 2018). The third sorbent was coconut charcoal, which has been applied for
210 water quality monitoring in combination with Oasis HLB (Escher et al., 2014; Leusch et al., 2014).
211 The 1 µg/L LPS solution only had an effect in the NF-κB assay after SPE for the Oasis HLB
212 extracts (Figure 3), with no effect in the Strata-X or coconut charcoal extracts up to a REF of 185
213 and 138, respectively (Figure S2, Table S2). No effect was observed in the ultrapure water blanks
214 for all three SPE sorbents. All samples were run in duplicate, with an average $BEQ_{bio, extract}$
215 (water+LPS) for Oasis HLB of $7.31 \times 10^{-2} \pm 3.95 \times 10^{-3}$ µg_{LPS}/L, which gives 7.3% effect recovery
216 by SPE for *E. coli* LPS based on Equation 2.

217 As the same conditioning and elution solvents were used for all SPE sorbents, the lack of
218 effect recovery by Strata-X and coconut charcoal can be attributed to differences in the sorbent
219 properties. The LPS structure consists of hydrophilic carbohydrates, specifically a core
220 oligosaccharide and an O-antigen polysaccharide, and a hydrophobic lipid A region, with the
221 biological activity of LPS related to lipid A (Fung et al., 2017). Coconut charcoal is typically used
222 to extract hydrophilic contaminants (Stepien and Puttmann, 2013) and therefore was not able to
223 extract lipid A. The difference in recovery between Oasis HLB and Strata-X is unexpected as both
224 sorbents are similar and previous studies have found similar recoveries for micropollutants
225 (D'Archivio et al., 2007). While both Oasis HLB and Strata-X had the same sorbent mass (500 mg),
226 the particle size differed, with Oasis HLB having a particle size of 60 µm and Strata-X having a
227 particle size of 33 µm and this may have contributed to the observed difference. Given that Oasis
228 HLB was the only sorbent able to extract LPS to a limited extent, the rest of the study will focus
229 solely on Oasis HLB. This is also the SPE sorbent commonly used for the extraction of water
230 samples prior to bioanalysis (e.g. Scott et al., 2014; Suzuki et al., 2015; Hebert et al., 2018). While
231 optimizing LPS recovery was not the aim of the current study, greater recovery of LPS may be
232 achieved using silica-based aminopropyl SPE sorbents (Fung et al., 2017).

233

234 3.2. *NF-κB* activity in surface water

235 All four surface water Oasis HLB extracts had a response in the NF- κ B assay. The EC_{IR1.5} and
236 BEQ_{bio, extract} values are provided in Table 1, with all concentration-effect curves shown in Figure
237 S3. The ultrapure blank did not have a response in the assay (Figure S3). The EC_{IR1.5} values of the
238 surface water samples were within the range of previously quantified EC_{IR1.5} values for surface
239 waters from Europe (Figure 1), with effects observed at REF 1.0 (e.g. the native sample) to REF
240 4.2. Of the studies included in Figure 1, only Hebert et al. (2018) and Nivala et al. (2018) used
241 Oasis HLB for sample enrichment. Escher et al. (2014) used a combination of Oasis HLB and
242 coconut charcoal, Neale et al. (2015a) used LVSPE with Chromabond HR-X, HR-XAW and HR-
243 XCW, König et al. (2017) used LVSPE with HR-X only and Neale et al. (2017b) used multi-layer
244 SPE with EnviCarb, a mixture of Strata X-CW, Strata X-AW and Isolute Env+, and Oasis HLB.

245 The surface water samples were characterised to better understand what components could
246 be contributing to the response. While metals can activate the NF- κ B response, they were not
247 analyzed in the current study as metals are not extracted by SPE. Of the fifty-four micropollutants
248 analyzed, only nine were detected in SW04, the sample collected from the creek downstream of a
249 drinking water reservoir. All analyzed chemicals were below the reporting limit for SW01, SW02
250 and SW03 (Table S1). The detected chemicals in SW04 included food additives acesulfame K and
251 caffeine and pharmaceuticals carbamazepine, erythromycin, gabapentin, oxazepam, temazepam,
252 tramadol and venlafaxine. All detected chemicals were reported to be inactive in the NF- κ B assay in
253 the US EPA ToxCast database (US EPA, 2015). Therefore, it appears that the analyzed
254 micropollutants are not contributing to the observed NF- κ B response. However, oxazepam, which
255 was detected at 0.05 μ g/L in SW04, was found to significantly inhibit NF- κ B activity in T24 cells at
256 1 μ g/L (Khalaf et al., 2009).

257 The endotoxin concentration in the unenriched surface water samples was measured using
258 the LAL assay. The calibration curve with the *E. coli* endotoxin standard is provided in Figure S4A.
259 As a control, ultrapure water with a LPS concentration of 1 μ g/L was measured, giving a
260 concentration of 7.3 EU/mL (Figure S4B). While a conversion factor of 1 ng to 10 EU is often

assumed, in actuality the conversion factor for endotoxins can vary from 1 ng to 4 EU to 1 ng to 17 EU (Anderson et al., 2007). SW04 had the highest endotoxin concentration at 130 EU/mL, with lower endotoxin concentrations found for SW01, SW02 and SW03 (53 to 69 EU/mL) (Table 1, Figure S4B).

DOC properties, specifically total organic carbon concentration and UV-vis absorbance at 254 nm, were measured in the unenriched surface water samples (Table 1). Absorbance at 254 nm is often used as a surrogate for organic carbon concentration, though it is mainly associated with aromatic functional groups (Korshin et al., 2009). The more responsive samples in the NF- κ B assay, SW03 and SW04, had higher total organic carbon concentrations and UV-vis absorbance than SW01 and SW02. DOC is co-extracted by Oasis HLB and will be present in the sample extract. To determine if DOC could be contributing to the effect in surface water, model DOC, HA and NOM, were run in the NF- κ B assay.

3.3. DOC can activate the NF- κ B response

Unenriched model HA and NOM both induced a response in the NF- κ B assay (Figure S5). To rule out any potential artefacts from DOC autofluorescence, $EC_{IR1.5}$ was calculated after background correction. $EC_{IR1.5}$ values of 42.0 and 117 mg_C/L for HA and NOM, respectively, were obtained after background correction (Table S3). This corresponds to an $EC_{IR1.5}$ of REF 3.50 and 9.74 for HA and NOM, respectively, after converting from mg_C/L to REF for a water sample containing 12 mg_C/L. Co-extracted DOC has previously been shown to interfere with an isolated enzyme assay (Neale and Escher, 2013), but not with cell-based assays run in agonist mode (Neale and Escher, 2014; Neale et al., 2015b). Hseu et al. (2014) found that synthetic HA activated the NF- κ B response in mouse macrophages at a concentration of 100 mg/L of HA, with reactive oxygen species (ROS) formation and protein kinase B activation by HA potentially inducing dissociation of the I κ B-NF- κ B complex.

286 To determine if DOC still had an effect after SPE, 12 mg_C/L of HA and NOM were enriched
 287 by Oasis HLB. Both HA and NOM SPE extracts had a response in the NF-κB assay, with an EC_{IR1.5}
 288 of REF 49.5 and 18.7, respectively (Table S4, Figure S5). While unenriched HA had a greater effect
 289 than NOM, the opposite was true after SPE, indicating that a greater fraction of NOM was co-
 290 extracted by Oasis HLB. Indeed, effect recovery by SPE was 52% for NOM, while only 7.1% of
 291 HA was co-extracted. Low recovery (<5%) of another reference HA, Aldrich HA, by Oasis HLB
 292 has previously been observed based on organic carbon measurements (Neale and Escher, 2013).
 293 Raeke et al. (2016) recently found poor recovery of higher molecular weight organic matter by
 294 Oasis HLB, which was attributed to stronger sorption to the sorbent and consequently incomplete
 295 elution. Therefore, the poor recovery of HA compared to NOM may be due to differences in
 296 molecular weight, with HA typically having a larger molecular weight than NOM (Pavlik and
 297 Perdue, 2015).

298 299 *3.4. Contribution of endotoxins and DOC to the NF-κB response*

300 While sample enrichment by SPE aims to remove matrix components, allowing bioassays to detect
 301 the mixture effects of organic micropollutants, we have shown that model LPS and DOC can be co-
 302 extracted by SPE and can activate the NF-κB response. To gain an understanding of the
 303 contribution of micropollutants, DOC and endotoxins to the NF-κB response in the surface water
 304 extracts, BEQ_{bio, extract} was compared to the calculated bioanalytical equivalent concentrations of the
 305 water extract stemming from detected chemicals (BEQ_{chem, extract}), DOC (BEQ_{DOC, extract}) and
 306 endotoxins (BEQ_{endotoxin, extract}). Assuming that the mixture concept of concentration addition
 307 applies, which can be assumed in a reporter gene assay (Escher et al., 2013), the BEQs of the
 308 individual components can be summed up in a mass balance (Equation 4).

$$\text{BEQ}_{\text{bio, extract}} = \text{BEQ}_{\text{chem, extract}} + \text{BEQ}_{\text{DOC, extract}} + \text{BEQ}_{\text{endotoxin, extract}}$$

309 (4)

310

311 Micropollutants were only detected in one sample and none were active in the NF-κB assay,
 312 so $BEQ_{chem, extract}$ did not contribute to $BEQ_{bio, extract}$.

313 $BEQ_{DOC, extract}$ was calculated based on the relative effect potency (REP_{DOC}) of unenriched
 314 model NOM compared to *E. coli* LPS and the predicted concentration of DOC (C_{DOC}) in the assay
 315 (Equation 5). C_{DOC} was calculated using the initial organic carbon concentration of the surface
 316 water and assuming that 52% of DOC was co-extracted by SPE. $BEQ_{DOC, extract}$ was able to explain
 317 only 4 to 11% of $BEQ_{bio, extract}$ (Figure 4, Table S5), so DOC only had a minor contribution to the
 318 overall $BEQ_{bio, extract}$.

319 There are some uncertainties associated to this calculation. $BEQ_{DOC, extract}$ was calculated
 320 based on the effect and SPE recovery of model NOM; however, the organic carbon properties of the
 321 surface water samples will differ from model NOM and therefore, the effect and SPE recovery of
 322 DOC in the surface water samples may also be different.

323

$$BEQ_{DOC, extract} = REP_{DOC} \times C_{DOC} = \frac{EC_{IR1.5} (LPS)}{EC_{IR1.5} (DOC)} \times C_{DOC}$$

324 (5)

325

326 To calculate $BEQ_{endotoxin, extract}$ the endotoxin concentration in units of EU/mL measured in
 327 the surface water prior to extraction was first converted to $\mu g_{LPS}/L$ to determine $BEQ_{endotoxin} (water)$.
 328 If we assume that the mixture of diverse endotoxins in surface water is equipotent to *E. coli* LPS,
 329 $BEQ_{endotoxin} (water)$ can be estimated using a conversion factor of 7.3, which was based on the
 330 EU/mL response of the 1 $\mu g/L$ LPS solution. $BEQ_{endotoxin, extract}$ was then calculated using Equation 6
 331 with $BEQ_{endotoxin} (water)$ and experimental *E. coli* LPS effect recovery by SPE (7.3%). In all
 332 samples, $BEQ_{endotoxin, extract}$ was higher than the $BEQ_{bio, extract}$, but was still within an order of
 333 magnitude (Figure 4, Table S5). This suggests that co-extracted endotoxins, not DOC or
 334 micropollutants, are responsible for the NF- response in the surface water SPE extracts.

$$BEQ_{\text{endotoxin, extract}} = BEQ_{\text{endotoxin (water)}} \times \text{LPS effect recovery by SPE}$$

(6)

There are also a number of uncertainties associated with the calculation of $BEQ_{\text{endotoxin, extract}}$, which may lead to the estimation of $BEQ_{\text{endotoxin, extract}}$ being higher than $BEQ_{\text{bio, extract}}$. We assumed that SPE recovery was the same for all endotoxins as for LPS and that the relative effect potency of all endotoxins was similar to *E. coli* LPS. However, previous studies have found that LPS purified from other bacterial species and water samples can have very different inflammatory potencies (Dehus et al., 2006; Ohkouchi et al., 2015). Therefore, it is unlikely that *E. coli* LPS and surface water endotoxins were equipotent. Further, we have used a conversion factor of 7.3 for *E. coli* LPS, but previous studies have found that the conversion factor of 1 ng of environmental endotoxins can range from 4 EU to 17 EU (Anderson et al., 2007).

In addition, the mass balance approach does not take into account the presence of any undetected micropollutants in the surface water extracts that may inhibit the NF-κB response or the potential for any interactions between DOC and endotoxins, which could affect recovery or the biological effect. Despite these limitations, the similarity between $BEQ_{\text{endotoxin, extract}}$ and $BEQ_{\text{bio, extract}}$ suggests that co-extracted endotoxins can potentially explain a significant fraction of the effect of surface water extracts in the NF-κB assay, with co-extracted DOC having no substantial contribution.

4. Conclusions

Assays indicative of the NF-κB response have been applied for water quality monitoring (e.g. Neale et al., 2017b; Hebert et al., 2018), though there is limited understanding about the compounds driving the effect in SPE extracts. In this study, we have shown that model LPS and DOC can be co-extracted by SPE with Oasis HLB and that these extracts can induce a response in the NF-κB

360 assay. By characterising the endotoxin, micropollutant and organic carbon properties of four surface
361 water samples and measuring the effect of the extracts in the NF- κ B assay, it appears that
362 micropollutants do not contribute to the NF- κ B response in the samples, with no micropollutants
363 detected in three of the water samples despite substantial activity and none of the micropollutants
364 quantified in the other sample being active in the NF- κ B assay.

365 By comparing $BEQ_{\text{bio, extract}}$ with $BEQ_{\text{chem, extract}}$, $BEQ_{\text{DOC, extract}}$ and $BEQ_{\text{endotoxin, extract}}$, it
366 appears that co-extracted endotoxins have a major contribution to the NF- κ B response in surface
367 water extracts. While bioanalysis of environmental SPE extracts is typically expected to capture the
368 mixture effects of organic micropollutants, this study suggests that the NF- κ B assay can integrate
369 the effect of co-extracted endotoxins. Endotoxins can pose a risk to human health, with a recent
370 study finding an association between acute health effects and exposure to endotoxins in recreational
371 waters (Levesque et al., 2016). Therefore, the fact that the effects of co-extracted endotoxins are
372 captured by the NF- κ B assay is advantageous for water quality monitoring. However, alternate
373 extraction methods or using concentrated cell medium to analyze unenriched water samples may be
374 more suitable to evaluate endotoxin activity in the NF- κ B assay given the low LPS effect recovery
375 by Oasis HLB.

376 In contrast to assays indicative of specific modes of action, such as hormone receptor-
377 mediated assays, where the majority of causative chemicals are known, a wide range of chemicals
378 can have an effect in assays indicative of adaptive stress responses, with often only a small fraction
379 of effect explained by detected chemicals (Escher et al., 2013; Neale et al., 2015a). Consequently,
380 adaptive stress responses assays, such as the NF- κ B assay, are not recommended for water quality
381 monitoring alone, but as part of a test battery with assays indicative of different stages of the
382 cellular toxicity pathway.

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392

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560

561 **Figure Captions**

562

563 Figure 1: NF- κ B EC_{IR1.5} values from the literature for wastewater, surface water and drinking water
564 in units of relative enrichment factor (REF), along with EC_{IR1.5} values of surface water samples
565 from the current study. Studies that used Oasis HLB for sample enrichment are indicated with an
566 asterisk. ^aNivala et al. (2018); ^bEscher et al. (2014); ^cNeale et al. (2017b); ^dNeale et al. (2015a);
567 ^eKönig et al. (2017); ^fHebert et al. (2018).

568

569 Figure 2: Overview of the experimental approach in the current study, with known recovery of
570 pharmaceuticals and DOC by Oasis HLB SPE sorbent included. Samples where bioanalytical
571 equivalent concentrations (BEQ) were measured are indicated in red, while calculated bioanalytical
572 equivalent concentrations of the water extract stemming from endotoxins (BEQ_{endotoxin, extract}),
573 detected chemicals (BEQ_{chem, extract}) and DOC (BEQ_{DOC, extract}) are indicated in blue. ^aGros et al.
574 (2012); ^bNeale and Escher (2013).

575

576 Figure 3: Linear concentration-effect curves for the LPS solution extracts and the ultrapure water
577 blank after SPE with Oasis HLB in the NF- κ B assay.

578

579 Figure 4: Comparison of measured BEQ_{bio, extract} values with predicted BEQ_{endotoxin, extract} and
580 BEQ_{DOC, extract} values for the surface water samples. The contribution of BEQ_{chem, extract} was
581 negligible.

582 Table 1: Summary of the studied surface water samples and their effect in the NF-κB assay, endotoxin concentration, sum detected chemical
583 concentration and organic carbon properties.

Sample ID	Description	EC _{IR1.5} ± SE (REF)	BEQ _{bio, extract} ± SE (μg _{LPS} /L)*	Endotoxin units EU/mL ± SD	Sum chemical conc. (μM)	Total organic carbon (mg _C /L)	UV-vis (254 nm)
SW01	Drinking water reservoir, Northern Rivers Region	3.52 ± 0.15	(8.75 ± 0.52) × 10 ⁻²	69 ± 2.5	n.d	5.1	0.154
SW02	Drinking water reservoir, Gold Coast	4.22 ± 0.21	(7.29 ± 0.46) × 10 ⁻²	53 ± 0.30	n.d	5.9	0.108
SW03	Drinking water reservoir, Scenic Rim Region	1.25 ± 0.07	(2.46 ± 0.17) × 10 ⁻¹	57 ± 0.48	n.d	12	0.219
SW04	Creek downstream of drinking water reservoir, Brisbane	1.02 ± 0.06	(3.01 ± 0.20) × 10 ⁻¹	130 ± 9.8	2.57 × 10 ⁻³	9.0	0.275
Blank	Ultrapure water	>13.3	<2.31 × 10 ⁻²	0.08	n/a	n/a	0.000

584 *LPS EC_{IR1.5} = 0.31 ± 0.01 μg/L

585 SE: standard error; SD: standard deviation; n/a: not analyzed; n.d: not detected

Figure 1

Figure 2

Figure 3

Figure 4