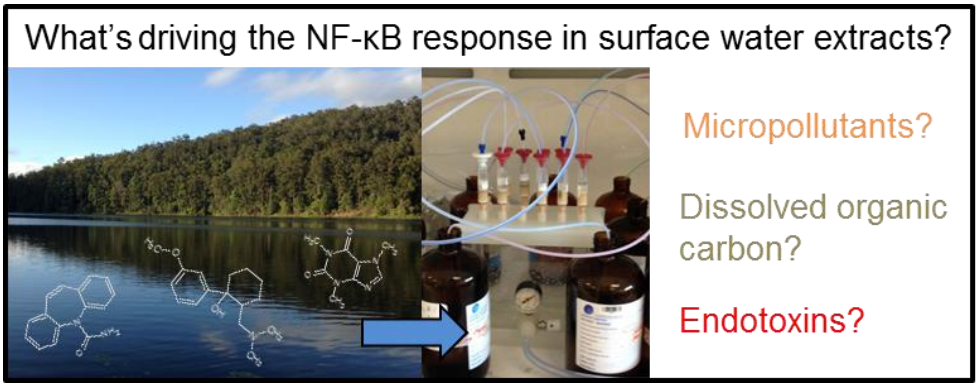
Graphical Abstract



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

Peta A. Neale[a,b]*, Frederic D.L. Leusch[a], Beate I. Escher[a,b,c,d]

[a]Australian Rivers Institute, School of Environment and Science, Griffith University, Southport QLD 4222, Australia

[b]The University of Queensland, Queensland Alliance for Environmental Health Sciences (QAEHS), Woolloongabba, Qld 4102, Australia

[c]UFZ – Helmholtz Centre for Environmental Research, 04318 Leipzig, Germany

[d]Eberhard Karls University Tübingen, Environmental Toxicology, Center for Applied Geoscience, 72074 Tübingen, Germany

Submitted to: Chemosphere

*Corresponding author: p.neale@griffith.edu.au

Abstract

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

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

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and Methods

2.1. Materials

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

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

2.2. LPS and DOC extraction

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

2.3. Surface water collection and enrichment for bioanalysis

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

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

2.4. Surface water characterisation

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

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

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

2.5. NF-κB assay

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

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

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

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

2.6. Data analysis

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

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

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

3.2. NF-κB activity in surface water

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

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

The endotoxin concentration in the unenriched surface water samples was measured using the LAL assay. The calibration curve with the *E. coli* endotoxin standard is provided in Figure S4A. As a control, ultrapure water with a LPS concentration of 1 µg/L was measured, giving a 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-κB 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-κB assay.

3.3. DOC can activate the NF-κB response

Unenriched model HA and NOM both induced a response in the NF-κB 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-κB 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 IκB-NF-κB complex.

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

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

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

$$BEQ_{bio,\ extract} = BEQ_{chem,\ extract} + BEQ_{DOC,\ extract} + BEQ_{endotoxin,\ extract} \quad (4)$$

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

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

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

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

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

$$BEQ_{endotoxin,\ extract} = BEQ_{endotoxin}\ (water) \times \text{LPS effect recovery by SPE} \quad (6)$$

There are also a number of uncertainties associated with the calculation of $BEQ_{endotoxin,\ extract}$, which may lead to the estimation of $BEQ_{endotoxin,\ extract}$ being higher than $BEQ_{bio,\ extract}$. 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 $BEQ_{endotoxin,\ extract}$ and $BEQ_{bio,\ extract}$ 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

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

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

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

Acknowledgements

This study was supported by the National Health and Medical Research Council (NHMRC) – European Union Collaborative Research Grant (APP1074775) and is part of the SOLUTIONS project (grant agreement number 603437), which is supported by the European Union Seventh Framework Programme. Shima Ziajahromi (Griffith University) is thanked for assistance with sample collection.

References

Ahn, K.S., Aggarwal, B.B., 2005. Transcription factor NF-kappa B - A sensor for smoke and stress signals. in: Kotwal, G.J., Lahiri, D.K. (Eds.). Natural Products and Molecular Therapy. Annals of the New York Academy of Science, pp. 218-233.

Anderson, W.B., Dixon, D.G., Mayfield, C.I., 2007. Estimation of endotoxin inhalation from shower and humidifier exposure reveals potential risk to human health. J. Water Health 5, 553-572.

APHA, 2012. Standard Methods for the Examination of Water and Wastewater, 22nd Edition. American Public Health Association, Washington, DC.

Baeuerle, P.A., Baltimore, D., 1988. IκB: A specific inhibitor of the NF-κB transcription factor. Science 242, 540-546.

D'Archivio, A.A., Fanelli, M., Mazzeo, P., Ruggieri, F., 2007. Comparison of different sorbents for multiresidue solid-phase extraction of 16 pesticides from groundwater coupled with high-performance liquid chromatography. Talanta 71, 25-30.

Dehus, O., Hartung, T., Hermann, C., 2006. Endotoxin evaluation of eleven lipopolysaccharides by whole blood assay does not always correlate with Limulus amebocyte lysate assay. J. Endotoxin Res. 12, 171-180.

El Marghani, A., Pradhan, A., Seyoum, A., Khalaf, H., Ros, T., Forsberg, L.H., Nermark, T., Osterman, L., Wiklund, U., Ivarsson, P., Jass, J., Olsson, P.E., 2014. Contribution of

411 pharmaceuticals, fecal bacteria and endotoxin to the inflammatory responses to inland waters. Sci.
412 Total Environ. 488, 228-235.
413 Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J.,
414 Denslow, N.D., Dopp, E., Hilscherova, K., Humpage, A.R., Kumar, A., Grimaldi, M., Jayasinghe,
415 B.S., Jarosova, B., Jia, A., Makarov, S., Maruya, K.A., Medvedev, A., Mehinto, A.C., Mendez, J.E.,
416 Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraish, F., Snyder,
417 S., Su, G.Y., Tang, J.Y.M., van der Burg, B., van der Linden, S.C., Werner, I., Westerheide, S.D.,
418 Wong, C.K.C., Yang, M., Yeung, B.H.Y., Zhang, X.W., Leusch, F.D.L., 2014. Benchmarking
419 organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays.
420 Environ. Sci. Technol. 48, 1940-1956.
421 Escher, B.I., Leusch, F.D.L., 2012. Bioanalytical tools in water quality assessment. IWA
422 Publishing, London.
423 Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M., Altenburger, R., 2013. Most oxidative stress
424 response in water samples comes from unknown chemicals: The need for effect-based water quality
425 trigger values. Environ. Sci. Technol. 47, 7002-7011.
426 Fung, F.M., Su, M., Feng, H.T., Li, S.F.Y., 2017. Extraction, separation and characterization of
427 endotoxins in water samples using solid phase extraction and capillary electrophoresis-laser induced
428 fluorescence. Sci. Rep. 7, 10774.
429 Gilmore, T.D., 2006. Introduction to NF-κB: players, pathways, perspectives. Oncogene 25, 6680-
430 6684.
431 Gros, M., Rodriguez-Mozaz, S., Barcelo, D., 2012. Fast and comprehensive multi-residue analysis
432 of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface
433 and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear
434 ion trap tandem mass spectrometry. J. Chromatogr. A 1248, 104-121.

Hebert, A., Feliers, C., Lecarpentier, C., Neale, P.A., Schlichting, R., Thibert, S., Escher, B.I., 2018. Bioanalytical assessment of adaptive stress responses in drinking water: A predictive tool to differentiate between micropollutants and disinfection by-products. Water Res. 132, 340-349.

Hseu, Y.C., Kumar, K.J.S., Chen, C.S., Cho, H.J., Lin, S.W., Shen, P.C., Lin, C.W., Lu, F.J., Yang, H.L., 2014. Humic acid in drinking well water induces inflammation through reactive oxygen species generation and activation of nuclear factor-κB/activator protein-1 signaling pathways: A possible role in atherosclerosis. Toxicol. Appl. Pharmacol. 274, 249-262.

Judson, R., Houck, K., Martin, M., Richard, A.M., Knudsen, T.B., Shah, I., Little, S., Wambaugh, J., Setzer, R.W., Kothya, P., Phuong, J., Filer, D., Smith, D., Reif, D., Rotroff, D., Kleinstreuer, N., Sipes, N., Xia, M.H., Huang, R.L., Crofton, K., Thomas, R.S., 2016. Analysis of the effects of cell stress and cytotoxicity on *in vitro* assay activity across a diverse chemical and assay space. Toxicol. Sci. 152, 323-339.

Jugan, M.L., Oziol, L., Bimbot, M., Huteau, V., Tamisier-Karolak, S., Blondeau, J.P., Levi, Y., 2009. *In vitro* assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants, rivers and drinking water supplies in the greater Paris area (France). Sci. Total Environ. 407, 3579-3587.

Khalaf, H., Salste, L., Karlsson, P., Ivarsson, P., Jass, J., Olsson, P.E., 2009. *In vitro* analysis of inflammatory responses following environmental exposure to pharmaceuticals and inland waters. Sci. Total Environ. 407, 1452-1460.

König, M., Escher, B.I., Neale, P.A., Krauss, M., Hilscherova, K., Novak, J., Teodorovic, I., Schulze, T., Seidensticker, S., Hashmi, M.A.K., Ahlheim, J., Brack, W., 2017. Impact of untreated wastewater on a major European river evaluated with a combination of *in vitro* bioassays and chemical analysis. Environ. Pollut. 220, 1220-1230.

Korshin, G., Chow, C.W.X., Fabris, R., Drikas, M., 2009. Absorbance spectroscopy-based examination of effects of coagulation on the reactivity of fractions of natural organic matter with varying apparent molecular weights. Water Res. 43, 1541-1548.

Leusch, F.D.L., Aneck-Hahn, N.H., Cavanagh, J.A.E., Du Pasquier, D., Hamers, T., Hebert, A., Neale, P.A., Scheurer, M., Simmons, S.O., Schriks, M., 2018. Comparison of *in vitro* and *in vivo* bioassays to measure thyroid hormone disrupting activity in water extracts. Chemosphere 191, 868-875.

Leusch, F.D.L., Khan, S.J., Laingam, S., Prochazka, E., Froscio, S., Trinh, T., Chapman, H.F., Humpage, A., 2014. Assessment of the application of bioanalytical tools as surrogate measure of chemical contaminants in recycled water. Water Res. 49, 300-315.

Levesque, B., Gervais, M.C., Chevalier, P., Gauvin, D., Anassour-Laouan-Sidi, E., Gingras, S., Fortin, N., Brisson, G., Greer, C., Bird, D., 2016. Exposure to cyanobacteria: acute health effects associated with endotoxins. Public Health 134, 98-101.

Maletz, S., Floehr, T., Beier, S., Klumper, C., Brouwer, A., Behnisch, P., Higley, E., Giesy, J.P., Hecker, M., Gebhardt, W., Linnemann, V., Pinnekamp, J., Hollert, H., 2013. *In vitro* characterization of the effectiveness of enhanced sewage treatment processes to eliminate endocrine activity of hospital effluents. Water Res. 47, 1545-1557.

Mehinto, A.C., Jia, A., Snyder, S.A., Jayasinghe, B.S., Denslow, N.D., Crago, J., Schlenk, D., Menzie, C., Westerheide, S.D., Leusch, F.D.L., Maruya, K.A., 2015. Interlaboratory comparison of *in vitro* bioassays for screening of endocrine active chemicals in recycled water. Water Res. 83, 303-309.

Miller, S.C., Huang, R.L., Sakamuru, S., Shukla, S.J., Attene-Ramos, M.S., Shinn, P., Van Leer, D., Leister, W., Austin, C.P., Xia, M.H., 2010. Identification of known drugs that act as inhibitors of NF-κB signaling and their mechanism of action. Biochem. Pharmacol. 79, 1272-1280.

Neale, P.A., Ait-Aissa, S., Brack, W., Creusot, N., Denison, M.S., Deutschmann, B., Hilscherova, K., Hollert, H., Krauss, M., Novak, J., Schulze, T., Seiler, T.B., Serra, H., Shao, Y., Escher, B.I., 2015a. Linking *in vitro* effects and detected organic micropollutants in surface water using mixture-toxicity modeling. Environ. Sci. Technol. 49, 14614-14624.

Neale, P.A., Altenburger, R., Ait-Aissa, S., Brion, F., Busch, W., Umbuzeiro, G.D., Denison, M.S., Du Pasquier, D., Hilscherova, K., Hollert, H., Morales, D.A., Novak, J., Schlichting, R., Seiler, T.B., Serra, H., Shao, Y., Tindall, A.J., Tollefsen, K.E., Williams, T.D., Escher, B.I., 2017a. Development of a bioanalytical test battery for water quality monitoring: Fingerprinting identified micropollutants and their contribution to effects in surface water. Water Res. 123, 734-750.

Neale, P.A., Brack, W., Ait-Aissa, S., Busch, W., Hollender, J., Krauss, M., Maillot-Maréchal, E., Munz, N.A., Schlichting, R., Schulze, T., Vogler, B., Escher, B.I., 2018. Solid-phase extraction as sample preparation of water samples for cell-based and other *in vitro* bioassays. Environ. Sci.: Process. Impacts 20, 493-504.

Neale, P.A., Escher, B.I., 2013. Coextracted dissolved organic carbon has a suppressive effect on the acetylcholinesterase inhibition assay. Environ. Toxicol. Chem. 32, 1526-1534.

Neale, P.A., Escher, B.I., 2014. Does co-extracted dissolved organic carbon cause artefacts in cell-based bioassays? Chemosphere 108, 281-288.

Neale, P.A., Escher, B.I., Leusch, F.D.L., 2015b. Understanding the implications of dissolved organic carbon when assessing antagonism *in vitro*: An example with an estrogen receptor assay. Chemosphere 135, 341-346.

Neale, P.A., Munz, N.A., Ait-Aissa, S., Altenburger, R., Brion, F., Busch, W., Escher, B.I., Hilscherova, K., Kienle, C., Novak, J., Seiler, T.B., Shao, Y., Stamm, C., Hollender, J., 2017b. Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams. Sci. Total Environ. 576, 785-795.

Niss, F., Rosenmai, A.K., Mandava, G., Orn, S., Oskarsson, A., Lundqvist, J., 2018. Toxicity bioassays with concentrated cell culture media-a methodology to overcome the chemical loss by conventional preparation of water samples. Environ. Sci. Pollut. Res. 25, 12183-12188.

Nivala, J., Neale, P.A., Haasis, T., Kahl, S., Konig, M., Muller, R.A., Reemtsma, T., Schlichting, R., Escher, B.I., 2018. Application of cell-based bioassays to evaluate treatment efficacy of conventional and intensified treatment wetlands. Environ. Sci.: Water Res. Technol. 4, 206-217.

512 Novák, J., Vrana, B., Rusina, T., Okonski, K., Grabic, R., Neale, P.A., Escher, B.I., Macova, M.,
513 Ait-Aissa, S., Creusot, N., Allan, I., Hilscherová, K., 2018. Effect-based monitoring of the Danube
514 River using mobile passive sampling. Sci. Total Environ. 636, 1608-1619.
515 O'Toole, J., Sinclair, M., Jeavons, T., Leder, K., 2008. Alternative water sources and endotoxin.
516 Water Sci. Technol. 58, 603-607.
517 Oeckinghaus, A., Ghosh, S., 2009. The NF-κB family of transcription factors and its regulation.
518 Cold Spring Harb. Perspect. Biol. 1, a000034.
519 Ohkouchi, Y., Tajima, S., Nomura, M., Itoh, S., 2015. Inflammatory responses and potencies of
520 various lipopolysaccharides from bacteria and cyanobacteria in aquatic environments and water
521 supply systems. Toxicon 97, 23-31.
522 Pavlik, J.W., Perdue, E.M., 2015. Number-average molecular weights of natural organic matter,
523 hydrophobic acids, and transphilic acids from the Suwannee River, Georgia, as determined using
524 vapor pressure osmometry. Environ. Eng. Sci. 32, 23-30.
525 Raeke, J., Lechtenfeld, O.J., Wagner, M., Herzsprung, P., Reemtsma, T., 2016. Selectivity of solid
526 phase extraction of freshwater dissolved organic matter and its effect on ultrahigh resolution mass
527 spectra. Environ. Sci.: Process. Impacts 18, 918-927.
528 Rosenmai, A.K., Lundqvist, J., le Godec, T., Ohisson, A., Troger, R., Hellman, B., Oskarsson, A.,
529 2018. *In vitro* bioanalysis of drinking water from source to tap. Water Res. 139, 272-280.
530 Schroeder, A.L., Ankley, G.T., Houck, K.A., Villeneuve, D.L., 2016. Environmental surveillance
531 and monitoring - The next frontiers for high-throughput toxicology. Environ. Toxicol. Chem. 35,
532 513-525.
533 Scott, P.D., Bartkow, M., Blockwell, S.J., Coleman, H.M., Khan, S.J., Lim, R., McDonald, J.A.,
534 Nice, H., Nugegoda, D., Pettigrove, V., Tremblay, L.A., Warne, M.S.J., Leusch, F.D.L., 2014. An
535 assessment of endocrine activity in Australian rivers using chemical and *in vitro* analyses. Environ.
536 Sci. Pollut. Res. 21, 12951-12967.

537 Sen, R., Baltimore, D., 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by
538 a posttranslational mechanism. Cell 47, 921-928.
539 Simmons, S.O., Fan, C.Y., Ramabhadran, R., 2009. Cellular stress response pathway system as a
540 sentinel ensemble in toxicological screening. Toxicol. Sci. 111, 202-225.
541 Stalter, D., O'Malley, E., von Gunten, U., Escher, B.I., 2016. Fingerprinting the reactive toxicity
542 pathways of 50 drinking water disinfection by-products. Water Res. 91, 19-30.
543 Stepien, D.K., Puttmann, W., 2013. Simultaneous determination of six hydrophilic ethers at trace
544 levels using coconut charcoal adsorbent and gas chromatography/mass spectrometry. Anal. Bioanal.
545 Chem. 405, 1743-1751.
546 Suzuki, G., Sato, K., Isobe, T., Takigami, H., Brouwer, A., Nakayama, K., 2015. Detection of
547 glucocorticoid receptor agonists in effluents from sewage treatment plants in Japan. Sci. Total
548 Environ. 527, 328-334.
549 Tousova, Z., Oswald, P., Slobodnik, J., Blaha, L., Muz, M., Hu, M., Brack, W., Krauss, M., Di
550 Paolo, C., Tarcai, Z., Seiler, T.B., Hollert, H., Koprivica, S., Ahel, M., Schollee, J.E., Hollender, J.,
551 Suter, M.J.F., Hidasi, A.O., Schirmer, K., Sonavane, M., Ait-Aissa, S., Creusot, N., Brion, F.,
552 Froment, J., Almeida, A.C., Thomas, K., Tollefsen, K.E., Tufi, S., Ouyang, X.Y., Leonards, P.,
553 Lamoree, M., Torrens, V.O., Kolkman, A., Schriks, M., Spirhanzlova, P., Tindall, A., Schulze, T.,
554 2017. European demonstration program on the effect-based and chemical identification and
555 monitoring of organic pollutants in European surface waters. Sci. Total Environ. 601, 1849-1868.
556 US EPA, 2015. Interactive Chemical Safety for Sustainability (iCSS) Dashboard v2,
557 http://actor.epa.gov/dashboard/, Accessed 8th Jan 2018.
558 Wichmann, G., Daegelmann, C., Herbarth, O., Strauch, G., Schirmer, K., Wostemeyer, J.,
559 Lehmann, I., 2004. Inflammatory activity in river-water samples. Environ. Toxicol. 19, 594-602.

560

561 **Figure Captions**

562

563 Figure 1: NF-κB $EC_{IR1.5}$ 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. [a]Nivala et al. (2018); [b]Escher et al. (2014); [c]Neale et al. (2017b); [d]Neale et al. (2015a);
567 [e]König et al. (2017); [f]Hebert 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. [a]Gros et al.
574 (2012); [b]Neale 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-κB 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.

Table 1

Click here to download Table: Table 1.docx

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

Sample ID	Description	$EC_{IR1.5}$ ± SE (REF)	$BEQ_{bio,\ extract}$ ± SE ($\mu g_{LPS}/L$)*	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	$(8.75 \pm 0.52) \times 10^{-2}$	69 ± 2.5	n.d	5.1	0.154
SW02	Drinking water reservoir, Gold Coast	4.22 ± 0.21	$(7.29 \pm 0.46) \times 10^{-2}$	53 ± 0.30	n.d	5.9	0.108
SW03	Drinking water reservoir, Scenic Rim Region	1.25 ± 0.07	$(2.46 \pm 0.17) \times 10^{-1}$	57 ± 0.48	n.d	12	0.219
SW04	Creek downstream of drinking water reservoir, Brisbane	1.02 ± 0.06	$(3.01 \pm 0.20) \times 10^{-1}$	130 ± 9.8	2.57×10^{-3}	9.0	0.275
Blank	Ultrapure water	>13.3	$<2.31 \times 10^{-2}$	0.08	n/a	n/a	0.000

*LPS $EC_{IR1.5}$ = 0.31 ± 0.01 µg/L

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

Figure 1

Click here to download Figure: Figure 1.docx

Figure 1

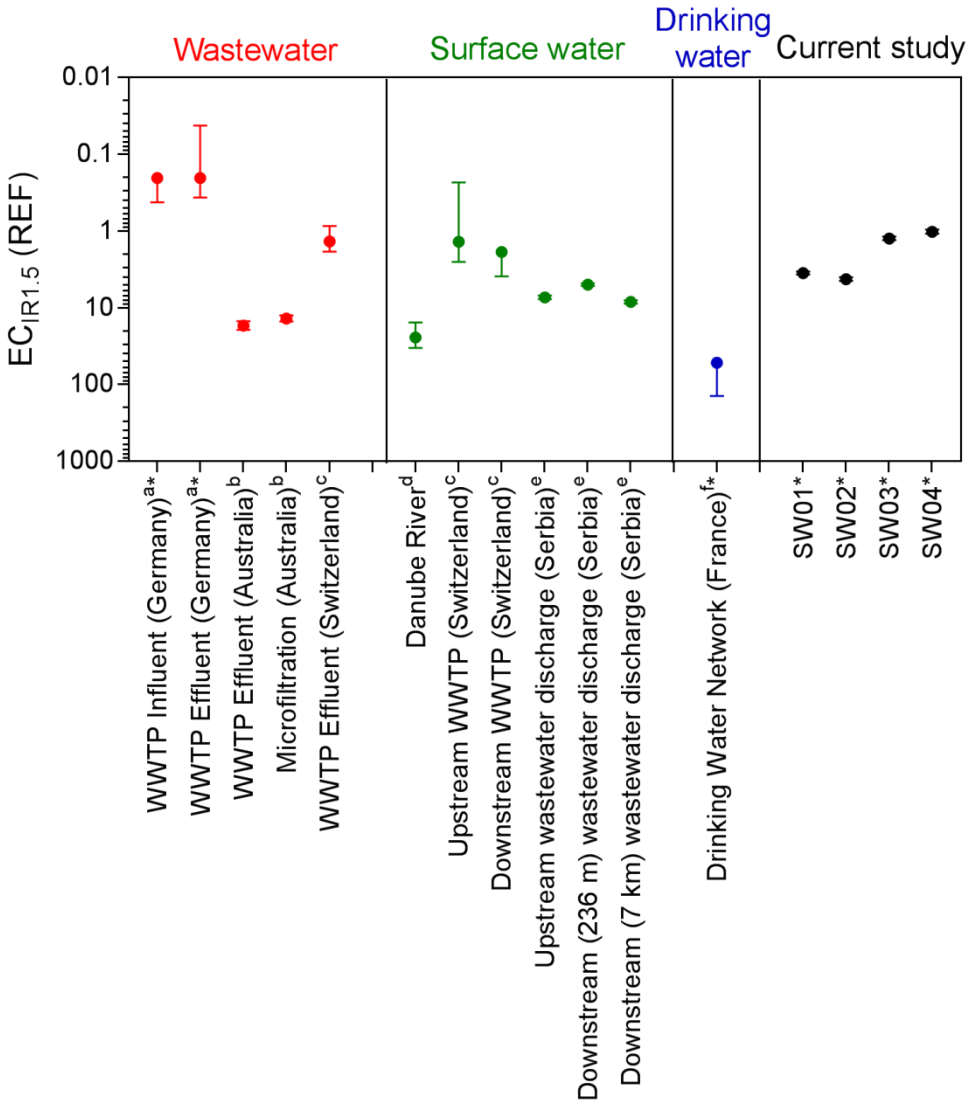


Figure 2
Click here to download Figure: Figure 2.docx

Figure 2

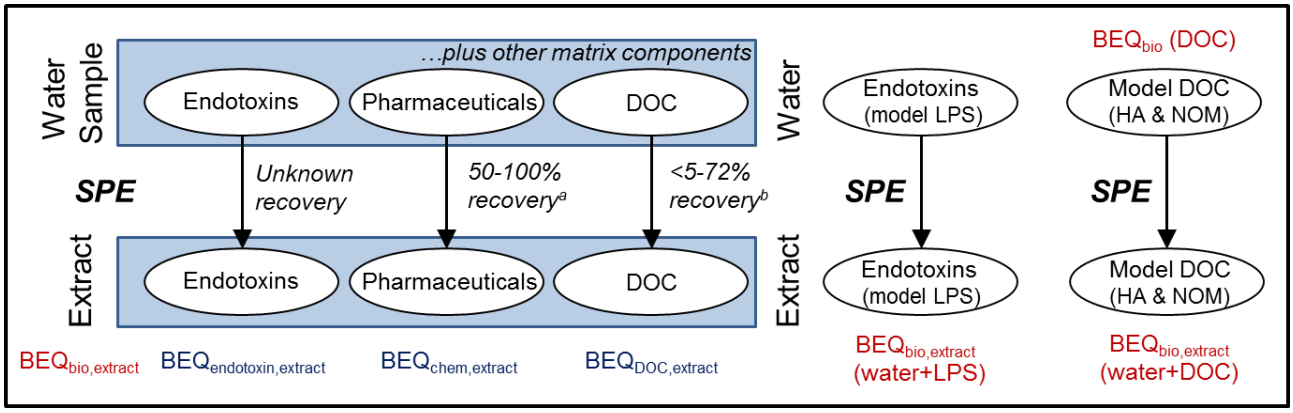


Figure 3

Click here to download Figure: Figure 3.docx

Figure 3

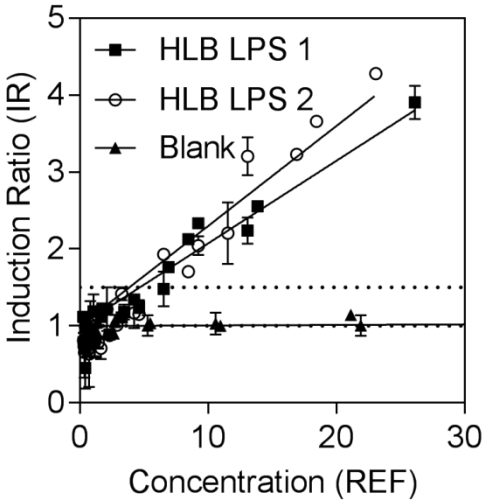
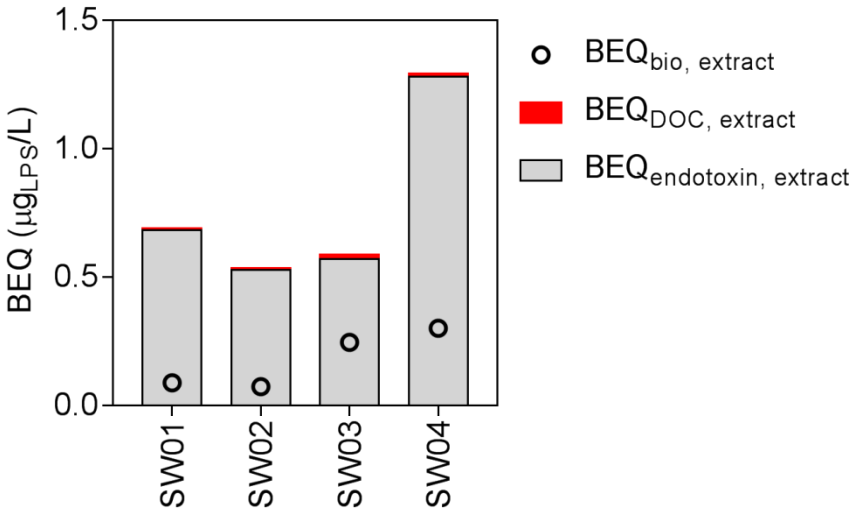


Figure 4

Click here to download Figure: Figure 4.docx

Figure 4



Supplementary Material

Click here to download Supplementary Material: Neale et al. Supplementary Material Revised.docx