

Bioanalytical Effect-Balance Model to Determine the Bioavailability of Organic Contaminants in Sediments Affected by Black and Natural Carbon

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1 Bioanalytical Effect-Balance Model to Determine the Bioavailability of Organic Contaminants in
2 Sediments Affected by Black and Natural Carbon

3

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24

25 **Abstract**

26 In sediments several binding phases dictate the fate and bioavailability of organic contaminants.
27 Black carbon (BC) has a high sorptive capacity for organic contaminants and can limit their
28 bioavailability, while the fraction bound to organic carbon (OC) is considered to be readily
29 desorbable and bioavailable. We investigated the bioavailability and mixture toxicity of sediment-
30 associated contaminants by combining different extraction techniques with *in vitro* bioanalytical
31 tools. Sediments from a harbour with high fraction of BC, and sediments from remote, agricultural
32 and urban areas with lower BC were treated with exhaustive solvent extraction, Tenax extraction
33 and passive sampling to estimate total, bioaccessible and bioavailable fractions, respectively.. The
34 extracts were characterized with cell-based bioassays that measure dioxin-like activity (AhR-CAFLUX)
35 and the adaptive stress response to oxidative stress (AREc32). Resulting bioanalytical equivalents,
36 which are effect-scaled concentrations, were applied in an effect-balance model, consistent with a
37 mass balance-partitioning model for single chemicals. Sediments containing BC had most of the
38 bioactivity associated to the BC fraction, while the OC fraction played a role for sediments with
39 lower BC. As effect-based sediment-water distribution ratios demonstrated, most of the bioactivity
40 in the AhR-CAFLUX was attributable to hydrophobic chemicals while more hydrophilic chemicals
41 activated AREc32, even though bioanalytical equivalents in the aqueous phase remained negligible.
42 This approach can be used to understand the fate and effects of mixtures of diverse organic
43 contaminants in sediments that would not be possible if single chemicals were targeted by chemical
44 analysis; and make informed risk-based decisions concerning the management of contaminated
45 sediments.

46

47 **Highlights**

- 48 (1) First study of black carbon-bound sediment contaminants with cell-based bioassays.
49 (2) Bioanalytical equivalents as a measure of biological effects in different media.
50 (3) Highest bioactivity of contaminants bound to black carbon.
51 (4) Mixture effects of waterborne chemicals were negligible.
52 (5) Effect-balance model to differentiate effect contribution of HOCs bound to OC and BC.

53 **Keywords (6):** *In vitro* bioassays, equilibrium passive sampling, black carbon, bioavailability, mixture
54 effect.

55 **1. Introduction**

56 **1.1 Bioavailability of organic contaminants in sediment**

57 Sediment contamination with hydrophobic organic contaminants (HOCs) is a worldwide problem and
58 risk assessment of contaminated sediments remains a challenge. It is widely recognized that sorption
59 of organic chemicals to different compartments within a sediment dictates their fate, availability and
60 thus also their toxicological risks. The effects caused by sediment-associated contaminants are
61 related to the bioavailable concentration rather than to the bulk chemical concentration (Hawthorne
62 et al., 2007; Lu et al., 2011; Lydy et al., 2014) and it has been proposed that the bioavailable
63 concentration of sediment-associated contaminants be considered in risk assessments of organic
64 chemicals (Ghosh et al., 2014; Ortega-Calvo et al., 2015).

65 Polymer-based partitioning can be used to determine the freely dissolved, bioavailable fraction of
66 sediment contaminants (Mayer et al., 2003; Cui et al., 2013; Mayer et al., 2014). Equilibrium-based
67 passive sampling using polydimethylsiloxane (PDMS) has been used widely (Difilippo and Eganhouse,
68 2010; Mäenpää et al., 2010; Bielská et al., 2014) and will also be applied in this study. Furthermore,
69 Tenax, a porous polymer resin, can be used as a sorptive phase for depletive extraction of sediment
70 contaminants. While Tenax is an infinite sink for sediment contaminants, it is often used to sample
71 the contaminant fraction that is easily and rapidly desorbed to the aqueous phase (Cornelissen et al.,
72 2001; Landrum et al., 2007). Together with freely dissolved pore water concentrations of
73 contaminants this makes up what is considered to be the bioavailable fraction of sediment
74 contaminants (Ortega-Calvo et al., 2015). The rapidly desorbing fraction has been shown to be the
75 main source of sediment pore water concentrations (Kraaij et al., 2002; You et al., 2007; Cui et al.,
76 2010) and it has been correlated to chemical residues in organisms (Kraaij et al., 2002; You et al.,
77 2007; Heijden and Jonker, 2009). A 24 h Tenax extraction has been used to accurately predict HOC
78 bioavailability from sediments (Cornelissen et al., 2001; You et al., 2011; Lydy et al., 2015).

79 Hydrophobic organic contaminants have a high affinity to the total organic carbon (TOC) in
80 sediments, which is made up of two major binding entities: amorphous organic carbon (OC) and
81 black carbon (BC). The OC consists of biogenic origin carbon and the BC consists of highly condensed
82 carbonaceous by-products and residues of incomplete combustion of fossil fuels and vegetation
83 such as soot, coal and kerogen (Goldberg, 1986; Schmidt and Noack, 2000). Organic carbon makes
84 up the bulk of TOC and often OC-normalized sediment contaminant concentrations have been
85 reported according to the equilibrium partitioning theory (Di Toro et al., 1991). In contrast, BC has
86 been shown to have much higher sorptive capacity than OC (Accardi-Dey and Gschwend, 2002;
87 Jonker and Koelmans, 2002; Cornelissen et al., 2005) and to strongly decrease the bioavailability of
88 sediment-associated contaminants (Knauer et al., 2007; Jia and Gan, 2014). Contaminants bound to
89 OC are generally more bioavailable than contaminants bound to BC as the partition constant
90 between OC and pore water (K_{OC}) can be several orders of magnitude smaller than the partition
91 constant between BC and pore water (K_{BC}) (Di Toro et al., 1991; Jonker and Koelmans, 2002;
92 Cornelissen et al., 2005). Therefore, OC is typically associated with the bioavailable fraction of
93 sediment contaminants, while BC is associated with the inaccessible fraction.

94 Studies investigating the bioavailability of sediment contaminants in relation to OC and BC sorption
95 have focussed on a few model compounds or chemical classes (Cornelissen and Gustafsson, 2003;
96 Brändli et al., 2008; Jia and Gan, 2014). Whereas, in the environment, contaminants are present in

97 complex mixtures and the observed toxicity of a given sample most likely stems from the mixture
98 effects. Bioanalytical tools allow the investigation of mixture toxicity of complex environmental
99 samples (Jahnke et al., 2016) and have in the past been used to investigate the toxicity of whole
100 sediment extracts. For example, mechanism-specific bioassays have been used to investigate the
101 estrogenic and dioxin-like potential of sediments by Houtman et al. (2006) and Jung et al. (2012)
102 while Kammann et al. (2004) used a combination of two toxicity assays to determine the genotoxic
103 and teratogenic potential of sediments. Li et al. (2013) and Vethaak et al. (2016) used a battery of
104 bioassays to investigate the toxicity and bioavailability of different chemical classes.

105 Here we apply bioanalytical tools, for the first time, to develop a comprehensive effect-balance of
106 various sediment types. We used a set of extraction techniques to differentiate the biological effects
107 of mixtures of chemicals associated with different compartments of the sediment (BC, OC and
108 water). The goal was to develop an effect-balance model to assign the mixture effect to the slowly
109 desorbing fraction associated with BC and to the readily desorbing fraction associated with OC. In
110 addition, the role of the pore water in relation to the solid sediment phases was investigated. We
111 compared a coal exporting harbour whose sediments contain relatively high fractions of BC with two
112 sites that are dominated by OC.

113 1.2 Theoretical considerations

114 The combined effects of a chemical mixture in an environmental sample derived from bioassays
115 have been expressed in bioanalytical equivalent concentrations (BEQ) (Escher and Leusch, 2012;
116 Escher et al., 2012). The ratio of the effect concentration of a reference compound (EC_{ref}) in a certain
117 bioassay to that of a sample is termed the BEQ_{bio} (Eq. 1). The EC_{ref} has units of $mol/L_{bioassay}$ while the
118 sample ECs (EC_{sample}) are given in sample mass equivalent transferred to the bioassay
119 ($kg_{sample}/L_{bioassay}$). The corresponding BEQs therefore have units of mol/kg_{sample} .

$$120 \quad BEQ_{bio} = \frac{EC_{ref}}{EC_{sample}} \quad (1)$$

121 The BEQ concept allows a comparison between the measured effect and the effects that a mixture
122 of known chemicals would elicit in the same bioassay. The BEQ_{chem} , derived from chemical analysis of
123 a sample, is defined as the sum of the measured concentrations of known chemicals i , C_i , multiplied
124 by their corresponding relative effect potencies (REP_i), which describe their effects in relation to a
125 reference compound (Eq. 2).

$$126 \quad BEQ_{chem} = \sum_{i=1}^n C_i \times \frac{EC_{ref}}{EC_i} = \sum_{i=1}^n C_i \times REP_i \quad (2)$$

127 If all bioactive chemicals are known and detected then $BEQ_{bio} = BEQ_{chem}$, but in reality there are
128 many unknown bioactive chemicals and consequently typically $BEQ_{bio} > BEQ_{chem}$ and the difference is
129 a measure of the effect caused by unknown chemicals. The advantage of using BEQ values as a
130 measure of effect is that they are expressed in concentration units of the reference compound and
131 thus they can be used in mass-balance models. However, the composition of the chemicals causing
132 the BEQs might vary between the different compartments (sediment, water, OC, BC) due to
133 differences in partition constants of different chemicals. Therefore partition constants related to
134 BEQs are not thermodynamic constants but operationally defined distribution ratios.

135 Compartments in a sediment-water-system and relationships between them as well as sampling
 136 phases relevant for this study are shown in Fig. 1. Equation 3 describes the mass-balance of chemical
 137 i in the sediment system ($n_{i, \text{sed}}$).

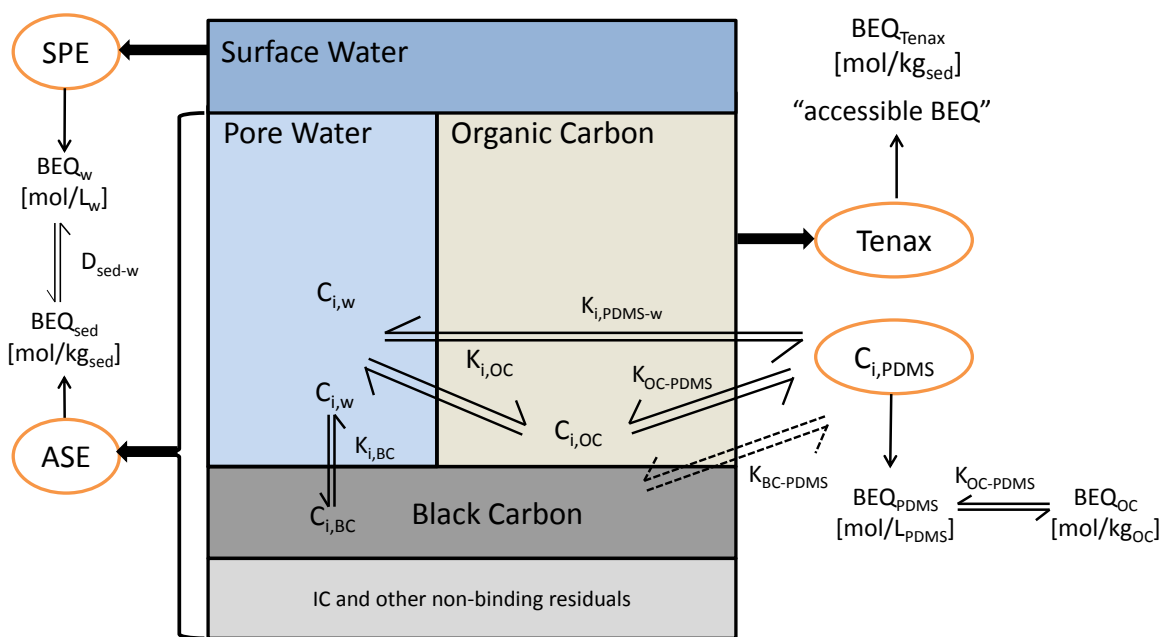
$$138 \quad n_{i, \text{sed}} = n_{i, \text{OC}} + n_{i, \text{BC}} + n_{i, \text{w}} + n_{i, \text{residual}} \quad (3)$$

139 $n_{i, \text{OC}}$ is the molar mass of i bound to OC and $n_{i, \text{BC}}$ is the molar mass of i bound to BC. The $n_{i, \text{w}}$ is the
 140 molar mass of i dissolved in the aqueous phase. The $n_{i, \text{w}}$ is the molar mass of i dissolved in the
 141 aqueous phase that is negligible in comparison to $n_{i, \text{OC}}$ and $n_{i, \text{BC}}$ for compounds with high K_{OC} and K_{BC}
 142 but it cannot be neglected for more hydrophilic chemicals. The $n_{i, \text{residual}}$ represents the amount of i
 143 bound to inorganic carbon and other ‘non-binding’ residuals. The amount of contaminants
 144 associated with this compartment is assumed to be negligible.

145 The total mass of the wet sediment ($m_{\text{sed}, \text{ww}}$) is the sum of the dry weight ($m_{\text{sed}, \text{dw}}$) and the pore
 146 water weight (m_{w} , or volume V_{w} , assuming a density of 1 kg L^{-1}), with the dry weight made up of OC,
 147 BC and residual solids (Eq. 4).

$$148 \quad m_{\text{sed}, \text{ww}} = m_{\text{sed}, \text{dw}} + m_{\text{w}} = m_{\text{OC}} + m_{\text{BC}} + m_{\text{residual}} + m_{\text{w}} \quad (4)$$

149



150

151 **Figure 1:** Conceptual model describing the relationships and equilibria in a sediment system. Orange
 152 circles represent extracts used in the study. Bold arrows \longrightarrow refer to exhaustive extractions, two-
 153 way arrows \rightleftharpoons refer to equilibrium partitioning, while dashed arrows indicate limited exchange
 154 between phases. BEQ = bioanalytical equivalent concentration, SPE = solid phase extraction, ASE =
 155 accelerated solvent extraction, PDMS = polydimethylsiloxane extraction, BC = black carbon, OC =
 156 organic carbon, IC = inorganic carbon.

157 Equation 3 can be translated into total sediment contaminant concentrations (on a wet weight basis)
 158 by using the fraction of OC and BC (f_{OC} and f_{BC}). These are calculated as the mass of OC or BC

159 respectively, divided by the total sediment mass including pore water (Eq. 5). The resulting mass-
 160 balance describes sorption of organic compounds to the two major sorption sites in sediments: OC
 161 and BC and the remaining aqueous fraction.

$$162 \quad C_{i,\text{sed},\text{ww}} = \left(\frac{m_{\text{OC}}}{m_{\text{sed},\text{ww}}} \right) \times \left(\frac{n_{i,\text{OC}}}{m_{\text{OC}}} \right) + \left(\frac{m_{\text{BC}}}{m_{\text{sed},\text{ww}}} \right) \times \left(\frac{n_{i,\text{BC}}}{m_{\text{BC}}} \right) + \left(\frac{m_{\text{w}}}{m_{\text{sed},\text{ww}}} \right) \times \left(\frac{n_{i,\text{w}}}{m_{\text{w}}} \right) \quad (5)$$

$$= f_{\text{OC},\text{ww}} \times C_{i,\text{OC}} + f_{\text{BC},\text{ww}} \times C_{i,\text{BC}} + f_{\text{w},\text{ww}} \times C_{i,\text{w}}$$

163 with the fractions f_y of compartment y related to the sediment wet weight (Eq. 6).

$$164 \quad f_{y,\text{ww}} = \frac{m_y}{m_{\text{sed},\text{ww}}} \quad (6)$$

165 For hydrophobic chemicals, the concentrations in the aqueous phase are negligible and the mass-
 166 balance reduces to Eq. 7 and fractions of wet sediment can be estimated as fractions of dry
 167 sediment (Eq. 8).

$$168 \quad C_{i,\text{sed},\text{dw}} = \left(\frac{m_{\text{OC}}}{m_{\text{sed},\text{dw}}} \right) \times \left(\frac{n_{i,\text{OC}}}{m_{\text{OC}}} \right) + \left(\frac{m_{\text{BC}}}{m_{\text{sed},\text{dw}}} \right) \times \left(\frac{n_{i,\text{BC}}}{m_{\text{BC}}} \right) = f_{\text{OC},\text{dw}} \times C_{i,\text{OC}} + f_{\text{BC},\text{dw}} \times C_{i,\text{BC}} \quad (7)$$

$$169 \quad f_{y,\text{dw}} = \frac{m_y}{m_{\text{sed},\text{dw}}} \quad (8)$$

170 and the $f_{y,\text{dw}}$ is related to $f_{y,\text{ww}}$ by Eq. 9.

$$171 \quad f_{y,\text{ww}} = \frac{m_{\text{sed},\text{dw}}}{m_{\text{sed},\text{ww}}} \times f_{y,\text{dw}} \quad (9)$$

172 Li et al. (2013) showed that PDMS can be used to sample the bioavailable fraction of sediment
 173 contaminants and that bioassays can be applied to determine the effects of this fraction.
 174 Furthermore, a theoretical partition constant between OC and PDMS, $K_{\text{OC-PDMS}}$, was derived by Li et
 175 al. (2013) that is independent of the hydrophobicity of the chemicals (Eq. 10) and can be used to
 176 convert $C_{i,\text{PDMS}}$ to $C_{i,\text{OC}}$ (Eq. 11) and $\text{BEQ}_{i,\text{PDMS}}$ to BEQ_{OC} for compounds sorbed to the OC fraction of a
 177 sediment using (Eq. 12).

$$178 \quad K_{i,\text{OC-PDMS}} = \frac{K_{i,\text{OC}}}{K_{i,\text{PDMSw}}} \approx K_{\text{OC-PDMS}} \quad (10)$$

$$179 \quad C_{i,\text{OC}} = K_{\text{OC-PDMS}} \times C_{i,\text{PDMS}} \quad (11)$$

$$180 \quad \text{BEQ}_{\text{OC}} = K_{\text{OC-PDMS}} \times \text{BEQ}_{\text{PDMS}} \quad (12)$$

181 The mixture effect of the total contaminants sorbed to a sediment ($\text{BEQ}_{\text{sed},\text{ww}}$) as well as the fractions
 182 sorbed to OC and BC can be expressed by Eq. 13. The fractions of OC and BC can be measured
 183 directly and the OC sorbed contaminants can be sampled from the sediment using PDMS

184 partitioning. The last unknown variable, the bioanalytical equivalent concentration of contaminants
 185 sorbed to BC (BEQ_{BC}), can then be derived from the BEQ-balance given in Eq. 13.

$$\begin{aligned}
 & \text{BEQ}_{\text{sed,ww}} = f_{\text{OC,ww}} \times \text{BEQ}_{\text{OC,ww}} + f_{\text{BC,ww}} \times \text{BEQ}_{\text{BC,ww}} + f_w \times \text{BEQ}_w \\
 186 & = f_{\text{OC,ww}} \times K_{\text{OC-PDMS}} \times \text{BEQ}_{\text{PDMS}} + f_{\text{BC,ww}} \times \text{BEQ}_{\text{BC,ww}} + f_w \times \text{BEQ}_w \quad (13) \\
 & \left[\frac{\text{kg}_{\text{OC}}}{\text{kg}_{\text{sed,ww}}} \times \frac{L_{\text{PDMS}}}{\text{kg}_{\text{OC}}} \times \frac{\text{mol}}{L_{\text{PDMS}}} + \frac{\text{kg}_{\text{BC}}}{\text{kg}_{\text{sed,ww}}} \times \frac{\text{mol}}{\text{kg}_{\text{BC}}} + \frac{L_w}{\text{kg}_{\text{sed,ww}}} \times \frac{\text{mol}}{L_w} \right]
 \end{aligned}$$

187 For hydrophobic chemicals Eq. 13 can be simplified to Eq. 14, as the fraction of HOCs in the water
 188 phase is essentially negligible.

$$\begin{aligned}
 189 & \text{BEQ}_{\text{sed,dw}} = f_{\text{OC,dw}} \times K_{\text{OC-PDMS}} \times \text{BEQ}_{\text{PDMS}} + f_{\text{BC,dw}} \times \text{BEQ}_{\text{BC,dw}} \left[\frac{\text{kg}_{\text{OC}}}{\text{kg}_{\text{sed,dw}}} \times \frac{L_{\text{PDMS}}}{\text{kg}_{\text{OC}}} \times \frac{\text{mol}}{L_{\text{PDMS}}} + \frac{\text{kg}_{\text{BC}}}{\text{kg}_{\text{sed,dw}}} \times \frac{L_w}{\text{kg}_{\text{BC}}} \times \frac{\text{mol}}{L_w} \right] \\
 190 & \quad (14)
 \end{aligned}$$

191 Assumptions made by this BEQ-balance (Eq. 14) are that: partitioning-based sampling with PDMS
 192 will sample contaminants that are mainly associated with the OC fraction of sediments and are in
 193 equilibrium with the pore water; and that the chemicals bound to BC do not partition to the PDMS.
 194 Most previous studies have implicitly made these assumptions, but since bioassays detect the
 195 mixtures of all chemicals the validity of this assumption can be checked.

196 1.3 Experimental approach

197 This work set out to experimentally test two hypotheses. The main assumption underlying the BEQ
 198 balances in Eq. 13 and 14 is that we can experimentally differentiate BEQ_{OC} and BEQ_{BC} by equilibrium
 199 partitioning to PDMS. To test this assumption we compare effects of PDMS and Tenax extracts.
 200 PDMS samples the equilibrium concentration in a non-depletive way while Tenax samples the water
 201 and OC bound chemicals in a depletive way. Depletive extraction could also be accomplished with
 202 larger volumes of PDMS (Smedes et al., 2013) but for a simpler experimental design we used Tenax
 203 according to Li et al. (2013). A 24 h Tenax extraction was applied to two sediments, one sediment
 204 containing next to no BC (<0.05%) and one containing an average fraction of BC (0.12%) for the
 205 sediments being studied. Our working hypothesis was that the bioavailable fractions extracted by
 206 the two methods would be the same for the sediment containing no BC, as all contaminants bound
 207 to OC are bioavailable, but would be different for the sediment containing appreciable amounts of
 208 BC.

209 We use two *in vitro* bioassays that target environmental pollutants at opposite ends of the
 210 hydrophobicity spectrum. Dioxin-like activity was quantified with the cell-based arylhydrocarbon
 211 receptor (AhR) chemically activated fluorescent expression (AhR-CAFLUX) assay (Nagy et al., 2002).
 212 Mainly hydrophobic chemicals activate the AhR. Oxidative stress, induced by more hydrophilic and
 213 reactive chemicals, was quantified by the activation of the adaptive stress response pathway. This
 214 pathway was probed with the AREc32 cell line (Wang et al., 2006) that measures the Nrf2-mediated
 215 activation of the antioxidant response element (ARE). Our hypothesis was that the full BEQ-balance
 216 (Eq. 13), including the water compartment, is required to describe the effects of mixtures of
 217 chemicals that cause oxidative stress. This is because many of the chemicals that induce oxidative
 218 stress are polar and thus typically have greater aqueous solubility. In contrast, the simplified dry

219 weight-based BEQ balance (Eq. 14) would be sufficient to describe the AhR assay because potent
220 ligands of AhR are typically moderate to very hydrophobic.

221 **2. Materials and Methods**

222 **2.1. Study area**

223 Gladstone Harbour is located in the central coast of Queensland, Australia south of the Tropic of
224 Capricorn (-23.829269, 151.250588). The harbour covers a total area of approximately 200 km² and is
225 sheltered by Curtis Island and Facing Island (Angel et al., 2010). Samples were taken from 14 sites in
226 the greater Gladstone Harbour area in June 2013, covering the harbour area and all major estuaries,
227 as well as four reference sites, chosen based on their distance from the main harbour area. A map
228 with the sampling sites and more information can be found in the Supporting Information (Fig. SI 1).
229 In order to compare the Gladstone Harbour sites (GH 1 to GH 14), classified as typical industrial sites,
230 further samples representing different land-uses were taken. One site from the Brisbane River (BR)
231 at the Oxley Creek outflow (-27.524, 152.994522) was sampled in October 2013, and two Fitzroy
232 River samples (FR 1, -23.37512, 150.51404, and FR 2, -23.53534, 150.88101, sampled during the
233 Gladstone sampling event) represents mainly urban areas. The Daintree River (DR, -16.251,
234 145.3067) was sampled in August 2013, representing a remote conservation area with minimal
235 agriculture (i.e., 6% agriculture – ABS, 2010).

236 **2.2 Sampling and sample preparation**

237 Grab samples of surface water (2 – 3 L per site) were collected in solvent-washed amber glass
238 bottles and were acidified to pH 3 with concentrated hydrochloric acid for sample preservation of
239 samples and to maximise extraction efficiency (Escher et al., 2005). Surface water samples were
240 filtered through a 1.6 µm glass fibre filter (GF/A, Whatman) to remove suspended solids and
241 subsequently extracted by solid phase extraction (SPE). Surficial sediment samples were collected
242 using a Van Veen grab sampler or with a stainless steel shovel if the site was accessible and stored in
243 solvent-washed glass jars (700 mL, 4 jars per site). In the laboratory the sediment samples were
244 homogenized in a stainless steel bowl. Approximately half of each sediment sample was preserved
245 with 0.1% NaN₃ (dry weight basis) and stored at 4 °C for subsequent passive sampling experiments,
246 while the other half was frozen at -20 °C until being freeze dried.

247 **2.3 Solid Phase Extraction (SPE) of water samples**

248 Water samples were extracted using SPE according to the methods described by Macova et al.
249 (2011). The extracted chemicals include freely dissolved chemicals and chemicals bound to dissolved
250 organic matter <1.6 µm (after filtration through a 1.6 µm glass fibre filter). Briefly, 2 – 3 L of surface
251 water sample were extracted using 1 g OASIS[®] HLB solid phase cartridges (Waters, Australia). A 2 L
252 aliquot of ultrapure water served as a process control and was treated in the same way as surface
253 water samples. The cartridges were conditioned in a Visiprep manifold with 10 mL acetone/hexane
254 (1/1), followed by 10 mL methanol and finally 10 mL of pH adjusted (pH 3) ultrapure water. The
255 surface water sample was percolated under vacuum and the cartridges were dried for 2 – 3 h (or
256 until dry). Dried cartridges were wrapped in aluminium foil and stored at -20 °C until elution. The
257 polar fraction of the sample was eluted from the cartridge with 10 mL methanol and the non-polar
258 fraction was eluted with 10 mL acetone/hexane (1/1). The solvents were percolated by gravity and
259 vacuum was applied to draw any remaining solvent into the receiving test tubes. All eluates were

260 evaporated to dryness under a nitrogen stream and redissolved in 40 μ L dimethyl sulfoxide (DMSO).
261 Samples were stored at -20 $^{\circ}$ C in amber HPLC vials until further use.

262 **2.4 Accelerated Solvent Extraction (ASE) of sediments**

263 Freeze-dried samples were extracted using accelerated solvent extraction (Thermo Scientific Dionex
264 ASE 350), following the standard U.S. EPA method 3545 (1995). Six grams of freeze-dried sediment
265 mixed with pre-cleaned diatomaceous earth were extracted in three static cycles, each lasting 5 min
266 at 100 $^{\circ}$ C and 1500 psi using acetone/hexane (1/1) as the extraction solvent. Flush-volume and
267 purge-time were 60% and 90 s, respectively. Process controls consisting of diatomaceous earth were
268 treated in the same way as samples. The extracts were blown down to dryness under a nitrogen
269 stream and redissolved in 60 μ L DMSO. Samples were stored in amber HPLC vials at -20 $^{\circ}$ C.

270 **2.5 Measurement of organic carbon and black carbon**

271 For carbon analysis, the samples were ground to a fine powder in a ringmill. Approximately 0.5 g of
272 the sample was weighed into a ceramic boat containing a nickel liner. Inorganic carbon (IC) was
273 removed from the samples with 5-6% H_2SO_4 and the sediment OC content was subsequently
274 measured by combustion on a LECO TruMac combustion analyser at 1300 $^{\circ}$ C. Black carbon was
275 measured using a chemo-thermal oxidation method (Gustafsson et al., 1997) in which first OC was
276 removed from the sample by oxidization at 375 $^{\circ}$ C in a muffle furnace for 18 h, followed by removal
277 of IC through acid digestion as described above. Black carbon was then also determined in the
278 sample by combustion on a LECO TruMAC combustion analyser at 1300 $^{\circ}$ C. Total carbon in the
279 sample is defined as the dry weights of the total organic carbon (TOC) and total inorganic carbon
280 (TIC) fractions (Eq. 15). Total organic carbon is defined as the sum of the amorphous organic carbon
281 (OC) and black carbon (BC) fractions (Eq. 16).

$$282 \quad f_{TC,dw} = f_{TOC,dw} + f_{TIC,dw} \left[\frac{g_{TC}}{g_{sed,dw}} \right] \quad (15)$$

$$283 \quad f_{TOC,dw} = f_{OC,dw} + f_{BC,dw} \left[\frac{g_{TOC}}{g_{sed,dw}} \right] \quad (16)$$

284 **2.6 Passive Sampling with PDMS**

285 Polydimethylsiloxane (PDMS; Shielding Solutions Limited) discs (thickness of 0.0762 mm, diameter of
286 16 mm, and density of 1.17 kg/L) were used to extract the bioavailable fraction of sediment-
287 associated contaminants. Only PDMS discs within the range of 21.71 mg \pm 5% were used. Before use
288 the PDMS discs were cleaned through sonication in methanol and acetone/hexane (1/1) for 30 min
289 each, consecutively.

290 To sample the bioavailable fraction of sediment contaminants, wet sediment equivalent to 20 g of
291 dry sediment was added to a 40 mL amber glass vial and brought up to a water content of
292 approximately 70% using water from the same sampling site. One PDMS disc was added to each vial,
293 keeping the sampler/sediment weight ratio at 0.001 for all sites. The amount of sediment used in the
294 experiments was chosen to ensure negligible depletion (< 5%) (Mayer et al., 2003), detailed
295 calculations can be found in the supplemental information (Section SI 2). Vials containing 20 mL

296 ultrapure water and a cleaned PDMS disc served as process controls. The vials were shaken at
297 150 rpm at room temperature. For each sediment sample, three replicate vials were prepared.

298 Equilibration times between the passive sampler and sediments with differing organic carbon
299 contents ($f_{OC,dw} = 0.3 - 2.7\%$) were determined by measuring bioassay based uptake curves, using an
300 established time-series method (Mayer et al., 2003; Smedes et al., 2013). Establishment of
301 equilibrium was determined by measuring bioanalytical equivalent concentrations in the AhR-
302 CAFLUX bioassay, which targets the effects of dioxin-like chemicals and is mainly triggered by very
303 hydrophobic compounds that have slow uptake kinetics. Li et al. (2013) and Jin et al. (2013) have
304 applied this method to establish time to equilibrium. The rationale behind applying BEQ is the
305 following: if the mixture effect of all chemicals is constant, one can assume that all bioactive
306 chemicals have reached equilibrium. Therefore the BEQ-based approach to assessing sampling
307 kinetics is as legitimate as using the more traditional approach of a series of chemicals that might be
308 present in some samples and not in others. Spiking a set of chemicals to samples is no alternative
309 because the kinetics is likely to be different in native and spiked sediments, as Langdon et al. (2013)
310 have shown in soils. An incubation time of 21 days was adequate, even for sediments with very low
311 organic carbon content (Section SI 3). The samples were shaken for 21 days after which the PDMS
312 discs were removed from the sediment, cleaned in ultrapure water, carefully wiped dry with lint-free
313 tissue to remove any adhering sediment particles (Li et al., 2013) and transferred to a clean vial.
314 PDMS discs were sonicated twice in 15 mL acetone/hexane (1/1), renewing and saving the solvent
315 between each sonication. The two solvent extracts were combined and blown down under a
316 nitrogen stream to dryness and redissolved in 40 μ L DMSO for direct application in the bioassays.
317 Excellent recovery of spiked PCDDs in lipid samples in previous work (Jin et al., 2013) confirmed the
318 suitability of this passive sampler extraction method.

319 **2.7 Tenax extraction**

320 Solid phase extraction with Tenax beads was carried out according to You et al. (2007) and adapted
321 by Li et al. (2013). Two sediments were chosen, sample DR with the lowest $f_{BC,TOC}$ ($< 2\%$) and GH 10
322 with an average $f_{BC,TOC}$ (14%). Wet sediment equating 1 g of dry sediment was added to a 40 mL
323 amber glass jar together with 0.25 g of Tenax resin (Sigma Aldrich; 11982). The jar was filled with
324 38 mL of ultrapure water and shaken at 150 rpm at room temperature for 24 h. Tenax was then
325 collected from the surface of the water, washed with ultrapure water and extracted twice in 15 mL
326 acetone/hexane (1/1). The extracts were combined, blown down to dry-ness and redissolved in
327 50 μ L DMSO for direct application in the bioassays. Tenax resin shaken in ultrapure water for 24 h
328 served as a process control.

329 **2.8 *In vitro* bioassays**

330 **2.8.1 AhR-CAFLUX**

331 Cells used for the assay are recombinant mouse hepatoma cells that were stably transfected with a
332 dioxin-responsive enhanced green fluorescent protein (EGFP) reporter gene (Nagy et al., 2002). The
333 cells respond to dioxins and dioxin-like chemicals that can bind to the AhR through the induction of
334 EGFP in a time-, dose- and chemical-specific manner. The AhR-CAFLUX bioassay was performed
335 according to Jin et al. (2013). Briefly, the cells were seeded in a black 96-well microtiter plate 24 h
336 prior to exposure to the extracts. The sample extracts were then dissolved in assay medium and
337 diluted serially (2-fold, 8 dilution steps) before adding to the assay plate. The samples were

338 incubated for 24 h at 33 °C, 5% CO₂ before relative fluorescent units (RFU) were measured by a
 339 FLUostar Omega plate reader (BMG Labtech). 2,3,7,8-TCDD served as a positive control and results
 340 were expressed as percentage of maximum 2,3,7,8-TCDD induction. The EC₅₀ values were derived
 341 from log-logistic dose-response curves and the results were expressed as 2,3,7,8-TCDD equivalent
 342 concentrations (TCDD-EQ).

343 2.8.2 AREc32

344 The AREc32 stable cell line (Wang et al., 2006) is based on MCF-7 breast cancer cells and measures
 345 Nrf2 mediated activation of the Antioxidant Response Element (ARE) via a luciferase reporter
 346 transgene. The assay was performed following the protocol described in Escher et al. (2013). Briefly,
 347 the cells were seeded in a white 96-well microtiter plate 24 h prior to dosing them with sample
 348 extracts. The extracts were serially diluted (2-fold, 8 dilution steps) in assay medium and added to
 349 the cells. After 24 h of incubation at 37 °C and 5% CO₂ the luciferase response was quantified after
 350 lysing the cells and then adding the luciferase reagent (20 mM Tricine at pH 7.8, 2.67 mM
 351 MgSO₄·7H₂O, 0.1 mM EDTA, 33.3 mM DTT, 261 μM Coenzyme A, 530 μM ATP, 470 μM luciferin
 352 (Promega Cooperation, USA) and MilliQ water; the solution's pH was adjusted to 7.8 before use). The
 353 luminescence was quantified immediately after addition of the luciferase reagent using a FLUostar
 354 Omega plate reader (BMG Labtech). t-Butylhydroquinone (tBHQ), a compound of moderate
 355 hydrophobicity (log K_{OW} = 2.57) which had been used before for water quality assessment (Escher et
 356 al., 2012), was chosen as a positive control and reference. The induction ratio (IR) of the luciferase is
 357 defined as the ratio of relative light units (RLU) of the sample divided by the average RLU of the
 358 controls (Escher et al., 2012). The assessment endpoint, EC_{IR1.5}, was derived from a linear
 359 concentration-IR regression through IR = 1. The resulting BEQs were related to tBHQ and expressed
 360 as tBHQ equivalent concentrations (tBHQ-EQ) (Escher et al., 2012).

361 2.8.3 Calculation of Relative Enrichment Factors

362 EC₅₀ or EC_{IR1.5} values, expressed in terms of the relative enrichment factor (REF) of the sample were
 363 derived from dose-response curves from both bioassays. The REF of the sample (Eq. 17) is the
 364 product of the dilution factor of the bioassay (Eq. 18) and the enrichment factor of the sample (Eq.
 365 19). The enrichment factor for the water (L_{water}/L_{extract}) as well as sediment (kg_{sed,ww}/L_{extract}) extracts
 366 are defined as the amount of water/sediment extracted by the final volume of the extract and were
 367 calculated according to Eq. 19. The dilution factor of each bioassay was calculated using Eq. 18.

$$368 \text{ REF} = \text{dilutionfactor}_{\text{bioassay}} \times \text{enrichmentfactor}_{\text{sample}} \quad (17)$$

$$369 \text{ dilutionfactor}_{\text{bioassay}} = \frac{\text{volumeofextractaddedtobioassay}}{\text{totalvolumeofbioassay}} \quad (18)$$

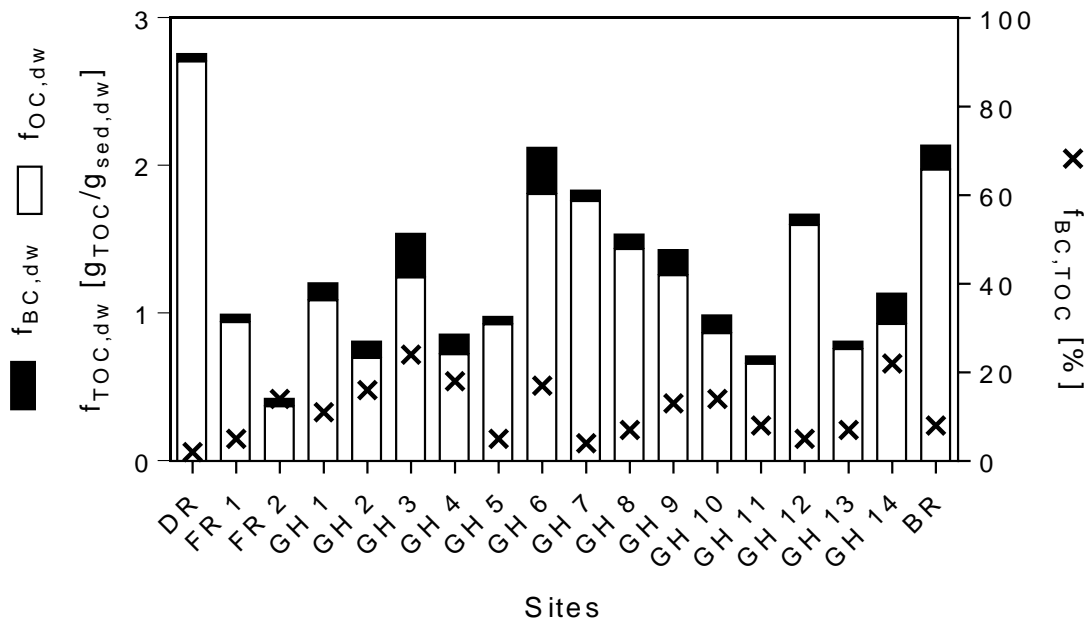
$$370 \text{ enrichmentfactor}_{\text{sample}} = \frac{V_w \text{ or } m_{\text{sed,ww}}}{V_{\text{extract}}} \quad (19)$$

371 Thus, BEQs are expressed as the amount of reference compound per litre of water for SPE extracts,
 372 per kilogram of wet weight sediment for sediment and Tenax extracts and per litre of PDMS for
 373 PDMS extracts.

374 **3. Results and Discussion**

375 **3.1 TOC and BC concentrations**

376 The fractions of TOC per dry-weight sediment, $f_{\text{TOC,dw}}$, ranged from 0.4 to 2.7%. The corresponding
377 fraction of BC, $f_{\text{BC,dw}}$, was < 0.05 to 0.3% (SI, Table SI 2). The BC constituted between < 2 to 24% of
378 the TOC of the samples, with the median being 9% (Fig. 2 and SI Table 2). Nine % BC content was
379 reported by Cornelissen et al. (2005) as a median of 300 literature values of sediments from around
380 the world. So, despite the fairly small sample size ($n = 18$) the investigated sites represent a wide and
381 representative variation of $f_{\text{BC,TOC}}$ (Fig. 2 and SI, Table 2). Although the 14 Gladstone Harbour (GH)
382 sites are geographically close to each other they exhibit highly variable BC/TOC ratios. This is likely to
383 be due to the natural heterogeneity of sediments as well as the diverse uses of adjacent land
384 including uses as a coal loading bay, shipping, recreation etc.



385 **Figure 2:** Total organic carbon content ($f_{\text{TOC,dw}}$) of sediment samples (bars, left y-axis) divided into
386 fractions of black carbon ($f_{\text{BC,dw}}$, solid shading) and organic carbon ($f_{\text{OC,dw}}$, no shading) as well as the
387 fraction of BC in TOC ($f_{\text{BC,TOC}}$) ('x', right y-axis).
388

389 **3.2 Equilibrium passive sampling of HOCs from sediment pore water**

390 The time to attain steady state for the passive sampler with the sediment slurry was determined for
391 five sediments with differing organic carbon contents ($f_{\text{OC,dw}} = 0.3 - 2.7\%$). Time to reach the steady
392 state ($t_{95\%}$) was 8 – 19 days, based on the AhR-CAFLUX bioassay. Details on passive sampling kinetics
393 can be found in the supplemental information (SI 3). Depletion of all sediments was below 5% for
394 contaminants active in the AhR-CAFLUX and AREc32 bioassays (SI, Table S3). With attainment of
395 steady state and non-depletion two important criteria of equilibrium passive sampling were fulfilled.

396

397 **3.3 Validation of BEQ-balance model assumptions**

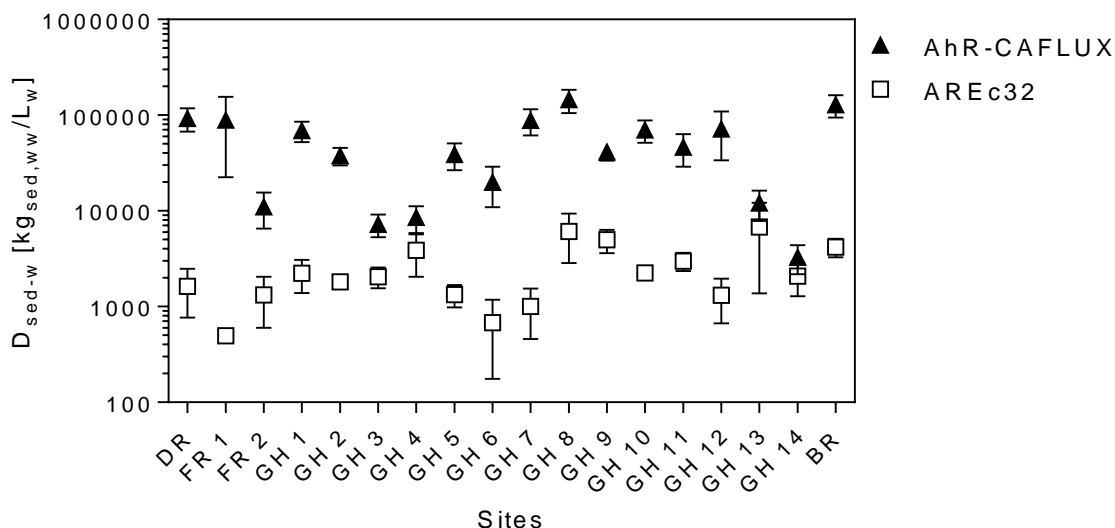
398 The main assumption for the simplified BEQ-balance model in Equation 14 is that the fraction of
 399 HOCs in the aqueous phase is low to negligible. This assumption can be tested by deriving an
 400 apparent BEQ-based distribution constant between HOCs in sediment (including pore water) and in
 401 the overlying water.

402 From the BEQ of the total sediment extracts (BEQ_{sed}) and the bioanalytical equivalents of the surface
 403 water samples (BEQ_w) a bioassay-based distribution constant (D_{sed-w}) was calculated (Eq. 20).

$$404 \quad D_{sed-w} \left[\frac{L_w}{kg_{sed,ww}} \right] = \frac{BEQ_{sed} \left[\frac{ng}{kg_{sed,ww}} \right]}{BEQ_w \left[\frac{ng}{L_w} \right]} \quad (20)$$

405 Surface water samples are not an ideal proxy of pore water, as they contain dissolved OC bound HOC
 406 fractions. In addition, organic contaminants in the water column and pore waters are very unlikely to
 407 be in chemical equilibrium with one another. Passive sampler-derived pore water concentrations
 408 would be a better proxy of sediment pore water concentrations, but due to the remoteness of the
 409 sampling locations such passive sampling was not possible.

410 High D_{sed-w} values, as observed for the AhR-CAFLUX bioassay (that ranged from 3300 (GH 14) –
 411 144,700 kg_{sed,ww}/L_w (GH 8); Fig. 3), indicate that the chemicals responsible for the measured effects
 412 were mostly very hydrophobic chemicals which partition into the sediment, leaving the BEQ in the
 413 water phase small to negligible. As expected, the D_{sed-w} values for the AREc32 bioassay were lower,
 414 at all sites, than those of the AhR-CAFLUX bioassay, ranging between 500 (FR 1) and 6800 kg_{sed,ww}/L_w
 415 (GH 13) (Fig 3). For the AREc32 bioassay it is thus possible that the measured effects were caused by
 416 both polar compounds and HOCs. Therefore, in the BEQ-balance model for the AREc32 bioassay, the
 417 water phase taken was into account (Eq. 13).



418
 419 **Figure 3:** Bioassay-based distribution constant between sediment and surface water (D_{sed-w}) values
 420 for the AhR-CAFLUX and AREc32 bioassays.

421 **3.4 Total bioaccessible vs. bioavailable fraction**

422 To investigate the differences between the bioavailable and the bioaccessible fractions samples DR
423 and GH 10 were chosen, as they represent two differing OC and BC contents. For the AhR-CAFLUX
424 bioassay, the rapidly desorbing fraction sampled from DR and normalized to OC was in the same
425 order of magnitude as the bioavailable fraction normalized to OC (Table 1, Tenax/PDMS ratio of
426 1.39). This reflects the very low level of BC (< 2%) and sorption of HOCs mainly to OC in this
427 sediment. For sample GH 10, with a BC content of 14%, the rapidly desorbed fraction was
428 approximately 13-fold higher than the bioavailable fraction (Table 1, Tenax/PDMS ratio of 12.6). This
429 shows that BC, in sample GH 10, is a relevant sorption phase for HOCs, making them less available
430 for equilibrium partitioning.

431 A comparison of BEQs of sample DR of bioaccessible sediment contaminants to total extractable
432 sediment contaminants showed that the total sediment contamination was bioaccessible (Table 1,
433 Tenax/ASE ratio of 0.99). In contrast, the bioaccessible fraction of sample GH 10 was smaller than
434 the total sediment BEQs (Tenax/ASE ratio of 0.11), indicating a high percentage of HOCs bound tightly
435 to BC, making them inaccessible in the short term.

436 The same extracts were also tested in the AREc32 bioassay, which monitors the adaptive stress
437 response pathway and is activated by chemicals that are mainly chemically reactive and more
438 hydrophilic than the chemicals that activate the AhR-CAFLUX bioassay (Martin et al., 2010; Escher et
439 al., 2012). No clear indication of different binding fractions could be found for the two investigated
440 sediments based on the AREc32 results. For both DR and GH 10 sediments the Tenax extractable
441 fraction was substantially greater than the PDMS extractable fraction, whereas the Tenax
442 extractable and the total extractable fractions were in the same order of magnitude. This indicates
443 that most of the chemicals extracted from these sediments were associated with the rapidly
444 desorbing fraction of the sediment and therefore accessible in a 24 h Tenax extraction. However,
445 interferences of the Tenax resin itself were detected in the process control and could not be
446 resolved through multiple cleaning steps of the resin before use, indicating that compounds in the
447 resin itself induce oxidative stress in the AREc32 bioassay. Therefore results for this bioassay should
448 be regarded with caution. Using the AREc32 bioassay on Tenax extracts is still useful as it highlights
449 that the AhR-CAFLUX bioassay is a better choice to investigate the binding of chemicals to BC and
450 that chemicals present in the water phase may play a more important role in the AREc32 bioassay.

451 **Table 1:** Comparison of PDMS, Tenax and ASE extractable AhR-CAFLUX and AREc32 BEQs for samples
 452 DR and GH 10. Mean values \pm standard deviation.

453

AhR-CAFLUX TCDD-EQ		DR	GH10	Process Control
				PDMS [$\mu\text{g}/L_{\text{PDMS}}$] Tenax [$\mu\text{g}/L_w$] ASE [$\mu\text{g}/\text{kg}_{\text{sed,dw}}$]
PDMS	[$\mu\text{g}/\text{kg}_{\text{OC}}$]	294 \pm 142	16 \pm 7	0.6 \pm 0.4
Tenax	[$\mu\text{g}/\text{kg}_{\text{OC}}$]	408 \pm 99	205 \pm 22	
Tenax	[$\mu\text{g}/\text{kg}_{\text{sed,ww}}$]	5.6 \pm 1.3	0.7 \pm 0.1	0.6 \pm 0.1
ASE	[$\mu\text{g}/\text{kg}_{\text{sed,ww}}$]	5.6 \pm 0.1	6.2 \pm 1.6	0.008 \pm 0.003
Ratio	Tenax/PDMS	1.39 \pm 0.75	12.6 \pm 5.5	
Ratio	Tenax/ASE	0.99 \pm 0.24	0.11 \pm 0.03	

AREc32 tBHQ-EQ		DR	GH10	Process Control
				PDMS [$\text{mg}/L_{\text{PDMS}}$] Tenax [mg/L_w] ASE [$\text{mg}/\text{kg}_{\text{sed,dw}}$]
PDMS	[$\text{mg}/\text{kg}_{\text{OC}}$]	266 \pm 47	340 \pm 68	92 \pm 12
Tenax	[$\text{mg}/\text{kg}_{\text{OC}}$]	703 \pm 33	4249 \pm 262	
Tenax	[$\text{mg}/\text{kg}_{\text{sed,ww}}$]	9.6 \pm 0.4	15.2 \pm 0.9	12 \pm 1
ASE	[$\text{mg}/\text{kg}_{\text{sed,ww}}$]	13 \pm 3.9	12.8 \pm 1.6	1 \pm 0.2
Ratio	Tenax/PDMS	2.6 \pm 0.5	13 \pm 2.6	
Ratio	Tenax/ASE	0.7 \pm 0.2	1.2 \pm 0.2	

454

455 3.5 BEQ-balance for hydrophobic contaminants (AhR-CAFLUX)

456 The BEQ_{sed} values of the exhaustive sediment extract in the AhR-CAFLUX bioassay were calculated
 457 for all 18 samples from the EC_{50} values (SI, Table SI 5) using Equation 1. The ww-normalised BEQ_{sed}
 458 values are listed in SI, Table SI 6. As compounds that induce the AhR are typically hydrophobic, the
 459 simplified mass-balance excluding the aqueous phase was applied and the $\text{BEQ}_{\text{sed,ww}}$ were converted
 460 to $\text{BEQ}_{\text{sed,dw}}$ (Fig. 4) using $f_{\text{sed,dw}}$ (SI, Table SI 2).

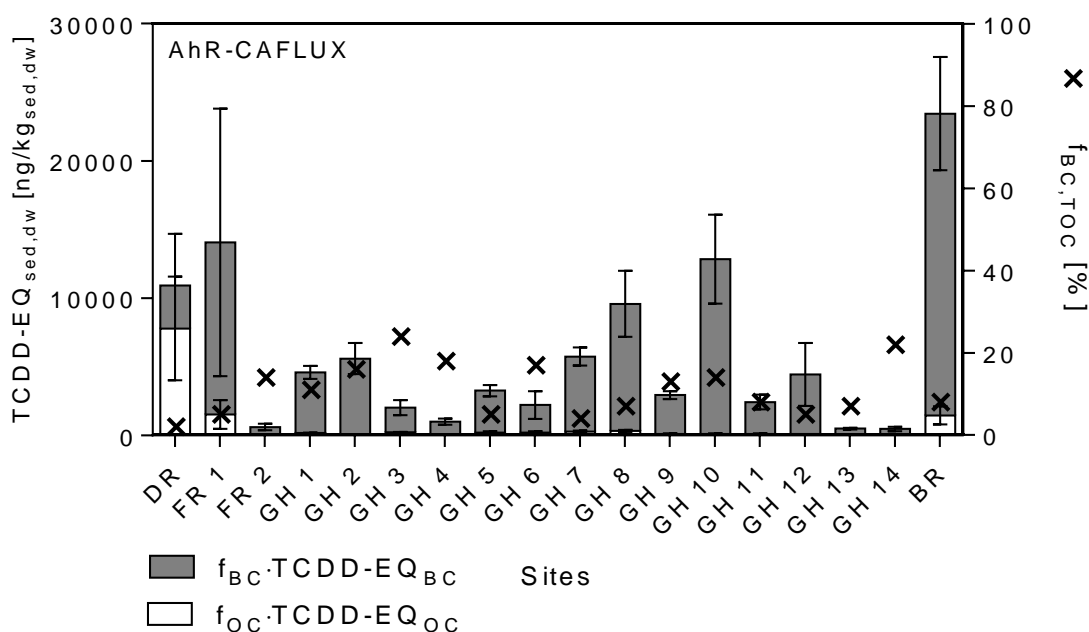
461 The contribution of organic carbon bound $f_{\text{OC}}\text{-BEQ}_{\text{OC}}$ to total $\text{BEQ}_{\text{sed,dw}}$ was calculated with the
 462 simplified mass-balance (Eq. 14) using the fraction of OC, the BEQs derived from the PDMS extract
 463 and the $K_{\text{OC-PDMS}}$ derived by Li et al (2013). This is depicted in Figure 4 as the white portion of the bar
 464 representing the TCDD- EQ_{sed} in the AhR-CALFUX bioassay. The contribution of the BC bound fraction
 465 $f_{\text{BC}}\text{-BEQ}_{\text{BC}}$ was calculated by subtracting BEQs contributing to the OC fraction from the total BEQs
 466 (black portion of the bars in Fig. 4).

467 There were clear differences between sites, with site BR (an estuary in vicinity to urban dwellings)
 468 showing the highest activity and the reference sites GH 13 and 14 showing the lowest overall
 469 activity. Overall GH sites showed lower AhR-CAFLUX activity compared to DR, FR 1 and BR. Sites
 470 GH 10, in the Calliope River, and GH 8, at the entrance to the marina, showed similar overall activity
 471 as site DR. The contribution of readily available, OC-bound, BEQs to the activity of the total sediment
 472 extract in the AhR-CAFLUX bioassay was markedly higher for site DR (71% of total BEQ, Fig. 4) than

473 all other sites investigated (1 – 12% of total BEQ). Consequently, $f_{BC} \cdot BEQ_{BC}$, the fraction of BEQs
474 bound to BC and unavailable for partitioning, was lowest for site DR (29%) and markedly higher (over
475 88%) for all other sites. Site DR contained the smallest amount of BC ($f_{BC,TOC} < 2\%$), thus leaving a
476 large portion of activating HOCs bound to OC and thus available for partitioning (Fig. 4). Site DR is a
477 remote site where the land-use is dominated by conservation with minimal agricultural activity, far
478 from industrial input, therefore having a low amount of BC. However, herbicides (Kroon et al., 2012),
479 pesticides (Magnusson et al., 2013) and unwanted pesticide by-products (Holt et al., 2008) may
480 contribute to the large AhR-CAFLUX effects found at this site. Furthermore, due to low BC content a
481 high amount of these activating compounds are available for partitioning and may thus cause
482 adverse effects on organisms in this catchment.

483 Site FR 1 showed higher activation of AhR compared to the river mouth of the same river, FR 2
484 (57.3 km downstream; Fig. 4), while the contribution of $f_{BC} \cdot TCDD-EQ_{BC}$ to total effect was comparable
485 for both sites (FR 1, 89%, and FR 2, 91%, Fig. 4). For most Gladstone Harbour sites more than 90% of
486 activating compounds appeared to be bound to BC and were thus unavailable for partitioning using
487 PDMS based equilibrium passive sampling. This was also the case for the reference sites GH 13 and
488 14, however, they showed overall lower total AhR-CAFLUX activity compared to the other GH sites.
489 Site BR, situated in a large urban estuary, showed the highest overall activity of the samples
490 investigated, but due to 0.2% BC at this site 94% of the total BEQs were not available for equilibrium
491 partitioning.

492 Braendli et al. (2008) have shown that small amounts of activated carbon can decrease the available
493 concentration of PAHs in soil/water suspensions by 99% and Lohmann et al. (2005) showed that BC
494 adsorption in harbour sediments was responsible for over 80 – 90% of the total PAH sorption and
495 more than 90% of the sorption of certain PCDDs in two different sediments. These results are
496 comparable to the harbour and estuary sediments investigated in this study (Figure 4), all of which
497 had detectable BC contents.



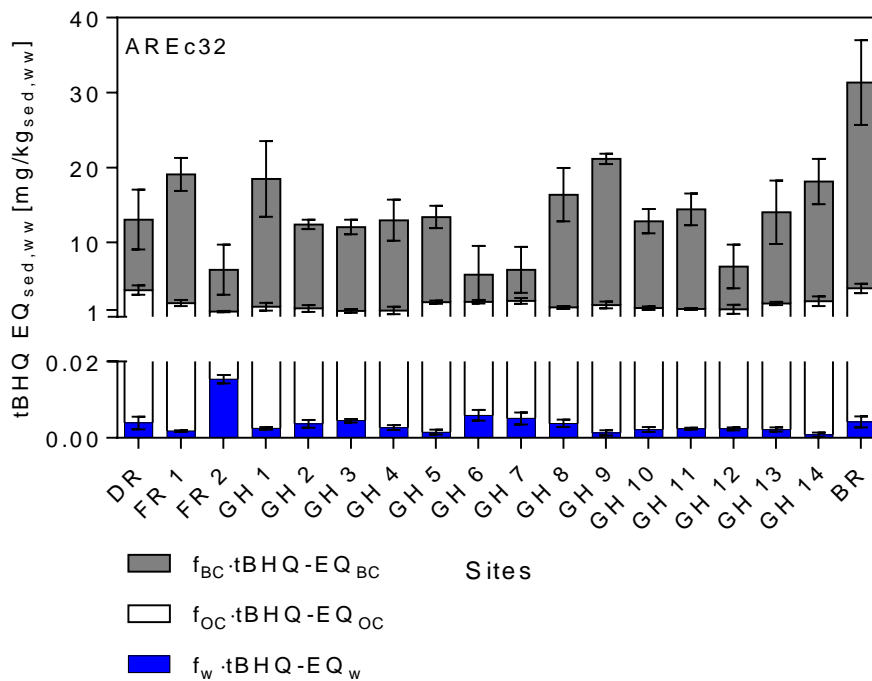
498 **Figure 4:** Bioanalytical equivalents of the exhaustive (ASE) sediment extract (TCDD-EQ_{sed,dw}) and
 499 contributions of organic carbon (OC) bound f_{OC} TCDD-EQ_{OC} (calculated from PDMS equilibrium
 500 sampling) and black carbon (BC) bound f_{BC} TCDD-EQ_{BC} (calculated with Equation 14) contaminants to
 501 total BEQs in the AhR-CAFLUX bioassay. The 'x' and right y-axis show the ratio of BC to OC, $f_{BC,TOC}$.
 502

503 3.6 BEQ-balance for more hydrophilic contaminants (AREC32)

504 Concentrations of HOCs have been shown to be close to equilibrium between sediment pore water
 505 and surface water in many instances, especially in well-mixed environments (van Noort and
 506 Koelmans, 2012), making surface water a good proxy for sediment pore water. The sites investigated
 507 in this study can be considered well-mixed and therefore BEQ_w was used as a proxy for the pore
 508 water BEQ in Eq. 13.

509 Our results showed that the contribution of the BEQ_w remained negligible in the overall mass-
 510 balance with contributions of BEQ_w being well below 1% for all sites (Fig. 5). Site FR 2 showed the
 511 highest contribution of BEQ_w (0.25%), despite showing one of the lowest toxicities. This could
 512 indicate fairly recent contamination or perhaps local discharge of activating compounds into the
 513 water phase at this site. The majority of chemicals that were bioactive in the AREC32 bioassay were
 514 associated with BC (64 – 93%) while 7 – 36% were associated with OC. The lowest contribution of BC
 515 associated BEQs ($f_{BC} \cdot BEQ_{BC}$) was for samples GH 6 and 7 (both 64%) while highest contributions were
 516 found for samples GH 3 and 4 (93%). Generally, the relative proportions of bioactive chemicals
 517 associated with BC and OC were similar for the AREC32 and the AhR-CAFLUX bioassays with most
 518 bioactivity associated with the BC of the sediment. However, for samples GH 6 and 7 around 64% of
 519 bioactive chemicals were associated with the BC, perhaps reflecting a higher percentage of polar
 520 compounds at these sites, which would be more readily available and associated with the OC. For
 521 sample DR the trend was reversed compared to the AhR-CAFLUX bioassay, with a higher percentage
 522 (72%) of bioactive compounds associated with the BC. Overall, it can be seen that the GH sites
 523 showed similar bioactivity in the AREC32 bioassay compared to the DT, FR and BR sites. This

524 difference between the GH sites was more pronounced for the more hydrophobic chemicals
 525 bioactive in the AhR-CALFUX bioassay.



526

527 **Figure 5:** Bioanalytical equivalents of the exhaustive sediment extract (tBHQ-EQ_{sed,ww}) and
 528 contributions of organic carbon bound ($f_{OC} \cdot tBHQ - EQ_{OC}$, calculated) and black carbon bound
 529 ($f_{BC} \cdot tBHQ - EQ_{BC}$, Equation 13) contaminants as well as water phase BEQs ($f_w \cdot tBHQ - EQ_w$, calculated) to
 530 total BEQs in the AREc32 bioassay.

531 3.7 Implications for sediment risk assessment

532 Results from this study showed that the overall toxicity of the whole sediment often poorly reflects
 533 the actual, bioavailable toxicity. The most contaminated site in this study, site BR that was
 534 influenced mainly by urban and industrial runoff, showed lower effects of available HOCs compared
 535 to the less overall polluted site DR, situated in a remote conservation area. However, lacking the
 536 influence of BC at the DR site, a higher proportion of the contaminants may be bioavailable,
 537 potentially posing a threat to the aquatic environment. In this context BC present in sediments, even
 538 at very low levels, can have a positive effect in reducing the bioavailability of HOCs to sediment-
 539 dwelling organisms. Previous studies have shown that reduction of the bioavailability of sediment
 540 contaminants through the presence of BC also decreased the risk of exposure to sediment dwelling-
 541 organisms, because it ultimately decreases the sediment pore water concentration of HOCs and
 542 uptake of these is believed to be mainly via the water phase (Cornelissen et al., 2005; Koelmans et
 543 al., 2006). This concept has also been used to artificially decrease bioavailability of HOCs by
 544 introducing manufactured carbonaceous materials to contaminated sediments (Zimmerman et al.,
 545 2004; Ghosh et al., 2011; Janssen and Beckingham, 2013), which in addition to natural carbonaceous
 546 materials further reduce the bioavailability of HOCs and contaminant flux into the water-column.
 547 Ortega-Calvo et al. (2015) proposed that when assessing the risks associated with contaminated
 548 sites the fraction of a chemical available for uptake into an organism within a given time span should

549 be considered. Using the methods described in this paper can give a good overview of bioavailability
550 of HOCs in the presence of differing carbon contents at contaminated sites and could help to make
551 informed management decisions.

552 **3.8 Conclusions**

553 Many soil and sediment guidelines still evaluate toxicity based on total contaminant concentration,
554 although it is widely accepted that total concentrations often show little or no relation to actual
555 toxicity to benthic organisms. This study showed that the bioavailability of bioactive sediment
556 contaminants was greatly decreased in the presence of BC. While this has been shown previously in
557 several other studies with selected individual compounds or compound classes, the novelty of this
558 study was to demonstrate this concept for a complex environmental sample, containing not only one
559 class of contaminants but a multitude of contaminants in mixtures and investigating the effects using
560 cell-based bioassays. A modelling approach was used to include BC-bound HOCs in combination with
561 *in vitro* bioassays to be able to compare the induction potential of bioavailable fractions as well as
562 slowly desorbing fractions. This method allows the differentiation of total contaminants and black
563 carbon-/organic carbon-bound contaminants and could potentially be applied when investigating
564 the hazard potential of sediments at industrially influenced sites with higher BC.

565 The benefit of using bioassays over chemical analysis for this evaluation is that the activity of an
566 unknown mixture of contaminants can be determined, while chemical analysis gives exact
567 concentrations but may miss contaminants contributing to the mixture effect. Especially when
568 dealing with diverse sources of contamination from agriculture, industry and mining as in this study,
569 the non-targeted bioanalytical tools can give a comprehensive picture of the contaminant burden
570 and will allow a prioritization of sites for further investigation and possibly identification of bioactive
571 pollutants. Mechanism-specific bioassays can furthermore give information about the chemical
572 classes present in a complex matrix such as sediments. A specific value of this work is the linkage to
573 the bioavailability of hydrophobic contaminants.

574 A range of factors including the type of BC and contaminants present, the presence of dissolved
575 organic carbon, temperature and pH may change the sorption to BC. Therefore, this framework can
576 only provide an initial estimate of the partitioning of contaminants in sediment. No concentrations
577 of contaminants were derived but effect-scaled concentrations (BEQs) and these provide valuable
578 insights on mixture toxicity of natural samples.

579

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754 **Figure Captions**

755 **Figure 1:** Conceptual model describing the relationships and equilibria in a sediment system. Orange
756 circles represent extracts used in the study. Bold arrows \longrightarrow refer to exhaustive extractions, two-
757 way arrows \longleftrightarrow refer to equilibrium partitioning, while dashed arrows indicate limited exchange
758 between phases. BEQ = bioanalytical equivalent concentration, SPE = solid phase extraction, ASE =
759 accelerated solvent extraction, PDMS = polydimethylsiloxane extraction, BC = black carbon, OC =
760 organic carbon, IC = inorganic carbon.

761 **Figure 2:** Total organic carbon content ($f_{\text{TOC,dw}}$) of sediment samples (bars, left y-axis) divided into
762 fractions of black carbon ($f_{\text{BC,dw}}$, solid shading) and organic carbon ($f_{\text{OC,dw}}$, no shading) as well as the
763 fraction of BC in TOC ($f_{\text{BC,TOC}}$) ('x', right y-axis).

764 **Figure 3:** Bioassay-based distribution constant between sediment and surface water ($D_{\text{sed-w}}$) values
765 for the AhR-CAFLUX and AREc32 bioassays.

766 **Figure 4:** Bioanalytical equivalents of the exhaustive (ASE) sediment extract (TCDD-EQ_{sed,dw}) and
767 contributions of organic carbon (OC) bound $f_{\text{OC}} \text{TCDD-EQ}_{\text{OC}}$ (calculated from PDMS equilibrium
768 sampling) and black carbon (BC) bound $f_{\text{BC}} \text{TCDD-EQ}_{\text{BC}}$ (calculated with Equation 14) contaminants to
769 total BEQs in the AhR-CAFLUX bioassay. The 'x' and right y-axis show the ratio of BC to OC, $f_{\text{BC,TOC}}$.

770 **Figure 5:** Bioanalytical equivalents of the exhaustive sediment extract (tBHQ-EQ_{sed,ww}) and
771 contributions of organic carbon bound ($f_{\text{OC}} \cdot \text{tBHQ-EQ}_{\text{OC}}$, calculated) and black carbon bound
772 ($f_{\text{BC}} \cdot \text{tBHQ-EQ}_{\text{BC}}$, Equation 13) contaminants as well as water phase BEQs ($f_{\text{w}} \cdot \text{tBHQ-EQ}_{\text{w}}$, calculated) to
773 total BEQs in the AREc32 bioassay.

774

775 **Table Captions**

776 **Table 1:** Comparison of PDMS, Tenax and ASE extractable AhR-CAFLUX and AREc32 BEQs for samples
777 DR and GH 10. Mean values \pm standard deviation.

778