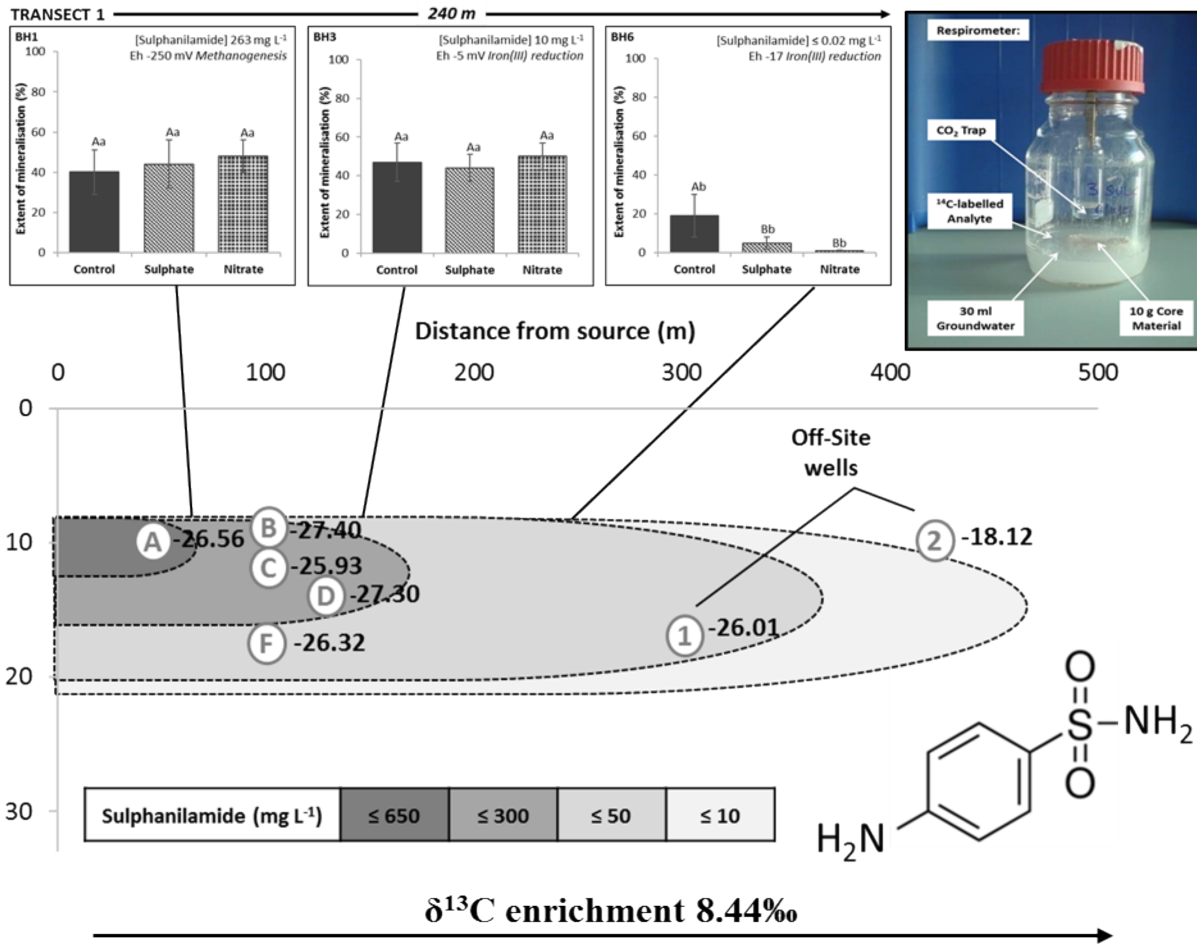

Extent of sulphanilamide biodegradation ~ 50 %
TRANSECT 1
240 m
BH1
[Sulphanilamide] 263 mg L-1
Eh -250 mV Methanogenesis
BH3
[Sulphanilamide] 10 mg L-1
Eh -5 mV Iron(III) reduction
BH6
[Sulphanilamide] ≤ 0.02 mg L-1
Eh -17 Iron(III) reduction
Extent of mineralisation (%)
Control
Sulphate
Nitrate
Respirometer:
CO2 Trap
14C-labelled Analyte
30 ml Groundwater
10 g Core Material
Distance from source (m)
Depth (mbs)
Off-Site wells
A -26.56
B -27.40
C -25.93
D -27.30
F -26.32
1 -26.01
2 -18.12
Sulphanilamide (mg L-1)
≤ 650
≤ 300
≤ 50
≤ 10
H2N
S
NH2
O
δ13C enrichment 8.44‰

Potential for natural and enhanced attenuation of sulphanilamide in a contaminated chalk aquifer

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KEYWORDS

Antibiotics; Sulphonamide; Groundwater contamination; Biodegradation; Stable isotope fractionation; IRMS.

ABSTRACT

Antibiotic compounds in the environment are of concern as they are biocidal and have the potential to drive antibiotic resistance in microbes. Understanding antibiotic biodegradation is important to the appreciation of their fate and removal from the environment. In this research an Isotope Ratio Mass Spectrometry (IRMS) method was developed to evaluate the extent of biodegradation of the antibiotic, sulphanilamide, in contaminated groundwater. Results

22 indicted an enrichment in $\delta^{13}C$ of 8.44‰ from -26.56 (at the contaminant source) to -18.12‰
23 (300 m downfield of the source). These results confirm reductions in sulphanilamide
24 concentrations (from 650 to 10 mg L^{-1}) across the contaminant plume to be attributable to
25 biodegradation (56 %) *vs.* other natural attenuation processes, such as dilution or dispersion
26 (42%). To understand the controls on sulphanilamide degradation *ex-situ* microcosms
27 assessed the influence of sulphanilamide concentration, redox conditions and an alternative
28 carbon source. Results indicated, high levels of anaerobic capacity (~50% sulphanilamide
29 mineralisation) to degrade sulphanilamide under high (263 mg L^{-1}), moderate (10 mg L^{-1}) and
30 low (0.02 mg L^{-1}) substrate concentrations. Nitrate and sulphate augmentation did not
31 significantly change the capacity of the groundwater to biodegrade sulphanilamide. Only
32 where alternative carbon sources were present did these augmentations alter sulphanilamide
33 biodegradation. Interestingly, these augmentations *decreased* sulphanilamide biodegradation
34 suggesting, under *in-situ* conditions, sulphanilamide could be acting as a nitrogen and sulphur
35 source. These findings are important as they highlight sulphanilamide being used as a carbon
36 and a putative nitrogen and sulphur source, under prevailing iron reducing conditions present
37 in the aquifer.

38 **1.0 INTRODUCTION**

39 Advances in analytical techniques have highlighted emerging organic contaminants, such as
40 pharmaceutical and personal care products, in multiple environmental media (Lapworth et al.,
41 2012, Pal et al., 2010). One particular concern is the occurrence of antibiotic compounds; as
42 these have the potential to interact with microorganisms in the environment and through this
43 interaction to perpetuate the development of antibiotic resistance (Kümmerer, 2009b). Thus,
44 there is concern that increasing levels of antibiotics, found in the environment, may promote
45 antibiotic resistance in microbes and potentially render antibiotics ineffective in treating

46 human and veterinary infections (Kümmerer, 2009b). It has been reported that between
47 100,000 and 200, 000 tons of antibiotics are used worldwide each year; with approximately
48 50 % used for human consumption and the remainder for animals, agriculture and
49 aquaculture (Kümmerer, 2009a). Due to their extensive use, these compounds are readily
50 released into the environment, from sources such as; wastewater treatment plants; hospital
51 effluents; livestock activities and manure application to soil, and; indirectly through ground
52 and surface water exchange (Lapworth et al., 2012, Michael et al., 2013, Rizzo et al., 2013).
53 Antibiotics and antibiotic resistant genes have been detected in wastewater discharges and
54 have been reported to persist in wastewater following its treatment (Michael et al., 2013).
55 Indeed, the biological processes employed at wastewater treatment plants have been reported
56 to promote the development and transfer of antibiotic resistant genes (Larcher and Yargeau,
57 2012, Michael et al., 2013).

58 Of the reported persistent pharmaceutical products, sulphonamides are widely detected in
59 groundwater across Europe (Lapworth et al., 2012), the United States of America (Barnes et
60 al., 2008) and China (Sui et al., 2015). Since the 1930s, over 5000 sulphonamide compounds,
61 (all derivatives of sulphanilamide) have been developed, with approximately 100 used as
62 antibiotics (Holm et al., 1995). The sulphonamide class of antibiotics can inhibit gram-
63 positive and gram-negative bacteria, as well as protozoa and as a consequence are among the
64 most frequently used antibiotics for human, veterinary and agriculture purposes (Brown,
65 1962, Liao et al., 2016, Larcher and Yargeau, 2012). It is estimated that 9.3 million kg of
66 antimicrobials are used annually in the USA, with 70 % used in animal feed as growth
67 promoters. In the UK, sulphonamides are the second most commonly used veterinary
68 antibiotic, making up 21 % of the annual consumption (448 000 kg) of antibiotics in the UK
69 (Sarmah et al., 2006).

70 Despite the anti-microbial properties of sulphonamides, studies suggest microbial
71 communities can adapt; with microbes developing resistance to the antibiotic becoming more
72 dominant and evolving to have the capacity to degrade antibiotics (Collado et al., 2013,
73 Herzog et al., 2013). Early work by Walker (1978) and Balba et al. (1979) demonstrated
74 sulphanilamide to be biodegradable. Sulphonamides have since been reported to degrade
75 under both aerobic (Collado et al., 2013, Drillia et al., 2005, Herzog et al., 2013, Larcher and
76 Yargeau, 2012, Liao et al., 2016, Müller et al., 2013, Reis et al., 2014, van Haperen et al.,
77 2001) and anaerobic (Carballa et al., 2007, Lin and Gan, 2011, Mohring et al., 2009)
78 conditions, and, in both soil and sediment environments (Baumgarten et al., 2011, Walker,
79 1978). Interestingly, microbes have been reported to utilise sulphonamides as a source of
80 carbon, nitrogen and/or sulphur, depending on the nutrient and environmental conditions they
81 are exposed to (Drillia et al., 2005, Herzog et al., 2013, Müller et al., 2013, Reis et al., 2014,
82 van Haperen et al., 2001).

83 However, there are limited accounts of sulphanilamide biodegradation in groundwater
84 environments and, information regarding their fate and degradation, as controlled by their
85 concentration and prevailing redox conditions, is very limited. Thus, new insight is needed
86 regarding how these controlling factors influence sulphonamide degradation. In addition, if
87 we are to engineer solutions to mitigate elevated concentrations of sulphonamides in the
88 environment, then we need a better understanding of how manipulation of electron acceptors
89 in groundwater might influence sulphonamide degradation.

90 The purpose of this research was to investigate the influence of sulphonamide concentration
91 and redox conditions on the sulphonamide biodegradation. Significantly, our research
92 focused on sulphonamide biodegradation in a contaminated chalk aquifer located below an
93 industrial facility (sulphanilamide concentrations $\leq$ 650 mg L^{-1}). This location provided

94 sampling transects that enabled the following controls on sulphanilamide degradation to be
95 evaluated: i) the interplay of sulphonamide concentration and redox condition, and ii) the
96 interplay of sulphonamide concentration, redox condition and the co-presence of alternative
97 carbon sources (specifically toluene). An Isotope Ratio Mass Spectrometry (IRMS) method
98 was developed to evidence carbon isotope fractionation during *in-situ* sulphanilamide
99 biodegradation. To assess the potential to enhance sulphonamide degradation, *ex-situ*
100 microcosms were supplemented with electron acceptors (sulphur and nitrogen) to evaluate
101 their influence on sulphonamide degradation.

102 2.0 MATERIAL AND METHODS

103 2.1 Site Description.

104 This research considered a chalk aquifer situated beneath a chemical plant in the United
105 Kingdom. The groundwater within the aquifer contained high levels of sulphanilamide (≤ 650
106 mg L^{-1}) and toluene (≤ 275 mg L^{-1}) (Figure 1). Partial degradation of these organic
107 compounds has exhausted dissolved oxygen in the aquifer and has given rise to anaerobic
108 conditions, dominated by Fe(III)- reduction (Eh values, reported in 69 sampled boreholes
109 across the site, from 270 to – 50 mV) (SI Figure 1) and sulphate-reduction (Eh values,
110 reported in 8 sampled boreholes, from 70 to – 130 mV) (SI Figure 1). Sulphanilamide is
111 mobile within the aquifer and its movement has resulted in the development of a solute plume
112 that extends approximately 300 m down gradient from the source zone (Figure 1 and 2), 10-
113 18 mbs (meters below surface). Across the plume sulphanilamide concentrations range from
114 1 to 133 mg L^{-1}, with movement of the plume being estimated at ≤ 0.01 m d^{-1} (Figure 1).
115 Beneath the “toluene works” (Figure 1), there exists a toluene plume, approximately 190 m
116 long, at 8-12 mbs, with concentrations ranging from 7 to 275 mg L^{-1}. The direction of
117 groundwater flow at the site is from a north to south-westerly direction (Figure 1 and 2). Thus

118 the sulphanilamide plume and toluene plume converge at approximately 140 m down
119 gradient of the sulphanilamide source zone (Figure 1), where an area of mixing exists.

120 **2.2 Chemicals.**

121 A radiolabelled analogue of sulphanilamide [ring-^{14}C(U)] was obtained from American
122 Radiolabelled Chemicals Inc., USA. Analytical grade (99%) acetone, methanol, ethanol,
123 sulphanilamide, sodium hydroxide (NaOH), and the salts, sodium sulphate (Na_2SO_4) and
124 sodium nitrate ($NaNO_3$), were obtained from Fisher Scientific, UK. The scintillation cocktail,
125 Ultima Gold™ XR, was obtained from Perkin Elmer Life & Analytical Sciences, UK. HPLC
126 mobile phase constituents (disodium hydrogen phosphate (KH_2PO_4) and *o*-phosphoric acid
127 (89 % w/w) (H_3PO_4) were obtained from Sigma Aldrich, UK.

128 **2.3 Aquifer core and groundwater sampling.**

129 Groundwater was collected from monitoring wells (n=19) across the study for use in the
130 IRMS method. The wells were purged and the stagnant water discarded before samples were
131 collected (Goldscheider et al., 2006). Groundwater was directed through a flow cell equipped
132 with an YSI 556 multi-parameter probe and collected in sterile polyethylene bottles (2 L).
133 The bottles were stored in the dark at 4 ^{o}C until use. During the construction of 6 new
134 boreholes at the site (Figure 2), cores were collected, using hollow stem auger techniques
135 (Chapelle, 1993) (Comachio MC305 drilling rig) from the saturated chalk zone, 10 mbs,
136 using PVC liners (0.1 m (*d*) by 0.50 m (*l*)), similar to that described by Johnson et al. (1998).
137 The PVC liners, containing the sampled cores, were capped and sealed following sampling
138 and kept in the dark at 4 ^{o}C until use. The boreholes were allowed to settle for 2 weeks prior
139 to groundwater sample collection.

140 **2.4 IRMS method.**

141 IRMS was used to quantify sulphanilamide biodegradation processes at the site and to
142 support the findings of the *ex-situ* microcosm studies (detailed in section 2.5). The method
143 was adapted from those described in the literature to determine biodegradation of dissolved
144 organic contaminants (United States Environmental Protection Agency, 2008), such as;
145 chlorinated solvents (Sherwood Lollar et al., 2000), aromatic petroleum hydrocarbons
146 (Griebler et al., 2003, Meckenstock et al., 1999, Mohammadzadeh et al., 2005) and fuel
147 oxygenates (Kolhatkar et al., 2002) in groundwater.

148 **2.4.1 Sample preparation using HPLC.**

149 To obtain concentrations of sulphanilamide, within detection limits for HPLC fractionation ($\leq$
150 0.2 mg ml^{-1}) and isotope analysis (1 nM L^{-1}) (United States Environmental Protection
151 Agency, 2008), the groundwater samples (1L) were pre-concentrated using freeze-drying
152 techniques (*Hetotrap CT60e* freeze-drying apparatus) (Castro and Garcia, 2002). The freeze-
153 dried portion was dissolved in acetone (10 ml) sonicated (10 min) and filtered (0.22 µm
154 PTFE syringe filters (Millex®)). Following filtration, the acetone was evaporated and the
155 dry sample dissolved in mobile phase (6.8 g L^{-1} disodium hydrogen phosphate (KH_2PO_4) in
156 Milli-Q water (18.2 MΩ.cm), reduced to pH 3 with *o*-phosphoric acid (89 % w/w) (H_3PO_4)
157 and the sulphanilamide collected via HPLC fractionation. The HPLC settings used a Gemini
158 column (5 µ C18 110A 150 x 2.00 mm 5 micron particle size (Phenomenex)), 25 µl injection
159 volume, 200 µl min^{-1} flow rate and an oven temperature 20 ^{o}C. A 25 µL injection of 0.1 mg
160 mL^{-1} [sulphanilamide] gave a signal of ~ 7 V and a retention time of 267 s. Optimum peak
161 amplitude and retention times for sulphanilamide were established using pure standards.

162 **2.4.2 Stable isotope analysis using an elemental analyser combined with IRMS.**

163 HPLC fractions were freeze-dried (described in section 2.4.1) and dissolved in acetone (1.5
164 ml), to isolate the sulphanilamide from the mobile phase. The acetone, was evaporated to 500

165 μL, transferred to a tin capsule (*Elemental Microanalysis, 13.5 x 8 mm, weight 82 mg, volume*
166 *679 μL*), where the remaining acetone was evaporated and the dried capsules crimped shut.
167 Using modifications of established IRMS methods (Mohammadzadeh et al., 2005, United
168 States Environmental Protection Agency, 2008), the capsules were analysed using an isotope
169 ratio mass spectrometer (*Delta XP, Conflow interface II, Thermo Finnigan, Bremen,*
170 *Germany*). The measured $^{12}C/^{13}C$ ratio was expressed relative to an international standard,
171 Vienna-PeeDee Belemnite (V-PDB), for carbon and reported using *delta* notation ($\delta^{13}C$) in
172 ‰ (*per mil*), see Equation [1] (Kelly et al., 1997, United States Environmental Protection
173 Agency, 2008, Sherwood Lollar et al., 2000).

174
$$\delta^{13}C\ (‰) = \left[\frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \right] X1000 \qquad [1]$$

175 Linearity and precision of the mass spectrometer were checked by measuring the peak
176 amplitudes and $\delta^{13}C$ values of sulphanilamide standards over a range of concentrations, from
177 0.2 to 0.8 mg (SI Figure 2 and 3).

178 Biodegradation was quantified using the δ^{13} values obtained for sulphanilamide in
179 groundwater samples from the site as detailed in SI Box 2 (United States Environmental
180 Protection Agency, 2008). In short, the fractionation factor (α), was calculated as $\alpha = R_a/R_b$,
181 where R is the stable isotope ratio ($^{13}C/^{12}C$) of sulphanilamide and the subscripts *a* and *b*
182 represent the source zone (monitoring well A) and down gradient monitoring points (off-site
183 well 2), respectively (Figure 3). The fraction remaining (f), was calculated using Equation 2:

184 $f = [R_b/R_a]^{1/(\alpha-1)}$ [2]

185 Biodegradation (B), was calculated as a percentage of material originally present using
186 Equation 3:

187 B = (1-*f*)*100 [3]

188 **2.5 Construction of *ex-situ* microcosms.**

189 Core material and groundwater were used to construct microcosms, similar to that described
190 by Johnson et al. (1998). The microcosms (n=3 per borehole) were prepared, to preserve
191 anaerobic integrity inside a nitrogen filled anaerobic glovebox. Each microcosm contained 10
192 g of core material and 30 ml of groundwater contained within a sterile Duran® bottle (250
193 mL). The microcosms were monitored using a ^{14}C-respirometery system as described by Reid
194 et al. (2005) to establish the competence of the microbial population to biodegrade ^{14}C-
195 labelled sulphanilamide. Thus, each microcosm was spiked with 2 kBq of ^{14}C-
196 sulphanilamide. CO_2 traps were prepared by adding 1 mL of NaOH (1 M) to a 7 ml glass
197 scintillation vial (Perkin Elmer Life & Analytical Sciences). The vials were suspended from
198 inside the Teflon coated Duran® bottle lid. Thereafter bottles were sealed using parafilm®
199 tape and placed on a shaker (110 rpm) in the dark, at 12°C (average groundwater temperature
200 at study site). At regular intervals the CO_2 traps were removed/replaced (under aseptic
201 anaerobic conditions) and Ultima Gold scintillation fluid (6ml) added. Traps were then
202 analysed for ^{14}C-activity (Packard Tri-Carb 2900TR Liquid Scintillation Analyser).

203 Manipulated treatment microcosms were constructed, for each of the boreholes, to establish
204 whether increasing the concentration of the electron acceptors; nitrate and sulphate, would
205 enhance the biodegradation of sulphanilamide. The additional microcosms (n=3 for each
206 borehole) were assembled, stored and monitored as described above. The treatment variants
207 were; denitrification (6.45×10^{-3} mol L^{-1} of NO_3 added as 548 mg L^{-1} $NaNO_3$) and; sulphate-
208 reduction (4.03×10^{-3} mol L^{-1} of SO_4 added as 572 mg L^{-1} Na_2SO_4). The calculations for the
209 nitrate and sulphate additions are provided in SI Box 1.

210 **2.6 Groundwater analytical methods.**

211 Sulphanilamide in groundwater samples was determined by HPLC, (Agilent 1100 diode array
212 detector (DAD1100) in series with a fluorescence detector (FLD1200). Settings; 100x3 mm
213 column, packed with Spherisorb® (S50DS1); flow rate 0.5 ml min^{-1}; injection volume 10 µl;
214 temperature 40 ^{o}C; detection was 254 nm at 88 s, and; mobile phase was 6.8 g L^{-1} disodium
215 hydrogen phosphate (KH_2PO_4) in Milli-Q water (18.2 MΩ.cm), reduced to pH 3 with *o*-
216 phosphoric acid (90 % w/w) (H_3PO_4)) Samples (1.0 g) and an internal standard (0.40 g
217 methyl p-hydroxybenzoate) were prepared in 100 ml water/methanol (HPLC grade) (50:50
218 v/v). 6 ml of prepared sample was transferred to a cylinder and 2 ml of *o*-phosphoric acid in
219 methanol (l5 % v/v) added and diluted to 100 ml with mobile phase.

220 Groundwater samples were analysed by Alcontrol UK Ltd (MCERTS accredited according to
221 ISO 17025). Standard methods were used to determine nitrate, sulphate(American Public
222 Health Association (APHA) American Water Works Association (AWWA) Water
223 Environment Federation (WEF), 1999), iron (II) (German Institute for Standardisation, 1981)
224 and Toluene (United States Environment Protection Agency, 1984) in the groundwater at the
225 site.

226 **2.7 Statistical Analysis.**

227 Analysis of variance tests were performed using SPSS v.18 to establish significant difference
228 ($p \leq 0.05$). Significant effects were compared using *post-hoc* tests and student t-tests ($p \leq$
229 0.05).

230 **3.0 RESULTS AND DISCUSSION**

231 **3.1 Identification/quantification of *in-situ* sulphanilamide biodegradation using IRMS.**

232 The isotopic signature for sulphanilamide (0.4 mg), measured in a sulphanilamide standard
233 reference material obtained from the study site (n = 23) gave an average $\delta^{13}C$ value of -27.04

234 $\pm$ 0.4 ‰. This represents good reproducibility and accuracy, with standard deviations being
235 typically ± 0.4 ‰ between measurements, which is comparable to stable isotope fractionation
236 values reported in the literature for other hydrocarbon contaminants (Sherwood Lollar et al.,
237 2007).

238 Figure 3 represents a 2D-cross-section of the study site, showing sulphanilamide
239 concentration and associated sulphanilamide isotope compositions. Although, sulphanilamide
240 concentrations between the source zone (monitoring well A, 650 mg L^{-1}) and the monitoring
241 well, situated approximately 150 m down gradient (monitoring well D, 261 mg L^{-1}) decreased
242 there was no observable change in the isotopic composition in this region of the site (Figure
243 3).

244 One factor which could account for the lack of an observable change in isotopic composition
245 could be recharging and/or mixing effects of fresh contaminants (Wilkes et al., 2008). At the
246 study site, abstraction wells are situated to the south and down gradient of the plume. It is
247 likely, during abstraction, groundwater from up gradient is drawn down gradient causing a
248 subsequent mixing of contaminants. Therefore, the lack of an observable isotopic shift does
249 not necessarily mean biodegradation is absent (Wilkes et al., 2008). In contrast,
250 sulphanilamide concentrations, along the same pathway, reduced from 650 to 10 mg L^{-1} at the
251 off-site well, situated approximately 400 m downgradient from the source zone (Figure 3,
252 monitoring wells A and 2). Here an enrichment in $\delta^{13}C$ was observed, with values increasing
253 by 8.44‰ from -26.56 to -18.12‰. This change in isotope composition evidences the
254 occurrence of biodegradation at the site.

255 The degree of biodegradation at the site was quantified, using the δ^{13} values for
256 sulphanilamide from the source zone (Figure 3, well A, -26.6 ‰) and a monitoring point
257 situated downgradient (Figure 3, well 2, -18.1‰), where sulphanilamide concentrations

258 reduced from 650 to 10 mg L^{-1} across the site. The degree of biodegradation (B) was
259 calculated at 56 % (SI Box1), with a fractionation factor (α) of 1.47, and fraction remaining
260 (*f*), 0.44 (United States Environmental Protection Agency, 2008).

261 Thus, the 98% decrease in sulphanilamide concentration from up- to down-gradient (i.e. from
262 650 to 10 mg L^{-1}) can be rationalised as 56 % attributable to biodegradation processes and the
263 remaining 42 % attributable to other natural attenuation processes, such as dilution or
264 dispersion (United States Environmental Protection Agency, 2008).

265 Evidence is widely available to support the use of IRMS to quantify biodegradation for
266 volatile contaminants in groundwater environments, e.g. chlorinated hydrocarbons (Imfeld et
267 al., 2008, Sherwood Lollar et al., 2000), BTEX compounds (Griebler et al., 2003,
268 Meckenstock et al., 1999) and methyl tert-butyl ether (Kolhatkar et al., 2002, Spence et al.,
269 2005). Yet, there are limited accounts for pharmaceutical products and in particular,
270 sulphonamide compounds.

271 Our results highlight large amounts of sulphonamide biodegradation, in particular
272 sulphanilamide (56 %), are possible, which is further substantiated by reports of
273 sulfamethoxazole (Reis et al., 2014) (≤ 99,1%) and sulfadiazine (Li and Zhang, 2010) (≤ 53.4
274 %) biodegradation, during wastewater treatment, and ≤ 50 % of sulfamethoxazole,
275 sulfamethazine and sulfadimethoxine biodegradation in surface water and sediments (Zhang
276 et al., 2013).

277 Of particular interest is the natural attenuation of sulphanilamide in groundwater, via
278 anaerobic biodegradation processes (56 %), reported here, in which no invasive intervention
279 to remediate was made. Furthermore, these results introduce IRMS as a suitable tool to
280 quantify the biodegradation of antibiotics in groundwater environments and so would be of
281 use to authorities tasked with identifying which natural attenuation processes i.e. dilution,

282 dispersion and/or biodegradation, are responsible for a contaminants observed loss/reduction
283 in natural and wastewater treatment systems.

284 **3.2 *Ex-situ* quantification of ^{14}C-sulphanilamide natural and enhanced biodegradation.**

285 The maximum extent of sulphanilamide biodegradation observed in the *ex-situ* microcosms
286 ranged from 40-50 % (BH1, 3 and 4, Figure 4, SI Figure 4), reducing to ≤ 28 % at BH2, 5
287 and 6 (Figure 4, SI Figure 4). With no significant different ($p \leq 0.05$), observed between the
288 anaerobic (control) microcosms at BH1 (methanogenesis) BH3 (sulphate reduction) and BH4
289 (Iron (III) reduction) (Figure 4, SI Figure 4) the observed reduction in the extent of
290 biodegradation does not appear to be the result of differences in redox potential. The same
291 can be said for the sulphanilamide concentrations, with no significant difference ($p \leq 0.05$)
292 observed between the anaerobic (controls) microcosms at BH1 (263 mg L^{-1}), BH3 (10 mg L^{-1}
293) and BH4 (≤ 0.02 mg L^{-1}) (Figure 4, SI Figure 4).

294 There were contradicting results for the sulphate and nitrate augmented treatments. At BH1-
295 3, augmentation resulted in no significant ($p \leq 0.05$) enhancement in the extent of
296 biodegradation compared with the anaerobic (control) (Figure 4, SI Figure 4). This suggests
297 the addition of electron acceptors (sulphate and nitrate), factors considered to limit
298 biodegradation if depleted (Haack and Bekins, 2000), had no positive influence on the
299 competence of microbes present at these locations to mineralise sulphanilamide (BH1-3).
300 Furthermore, despite sulphate levels being depleted in the BH1 area (30 mg L^{-1}, Figure 1, A
301 and B), the addition of > 500 mg L^{-1} of sulphate required for the complete biodegradation of
302 sulphanilamide as a carbon source (SI Box 1, Eq.5), did not enhance the extent of
303 biodegradation (Figure 4, SI Figure 4). Given these results, it is suggested that some other
304 factor may be limiting the extent of biodegradation in BH1. For example, a lack of available

305 nutrients, such as phosphorus (Bragg et al., 1994, Cooney et al., 1985, Das and Chandran,
306 2011).

307 In contrast, at BH4-6, there was a significant reduction in the extent of biodegradation
308 observed in the sulphate and nitrate augmented treatments compared with the anaerobic
309 control (Figure 4, SI Figure 4). Furthermore, at BH2, 5 and 6 the nitrate augmented
310 treatments resulted in significantly ($p \leq 0.05$) less sulphonamide mineralisation compared to
311 the sulphate augmented treatments (Figure 4). It is unlikely this reduction is associated with
312 the observed decrease in sulphanilamide concentration (≥ 10 mg L^{-1} at BH1-3 and ≤ 0.02 mg
313 L^{-1} at BH4-6) (Figure 4, SI Figure 4) as threshold concentrations usually indicate the start or
314 cessation of biodegradation rather than a reduction in the extent (Boethling and Alexander,
315 1979). Furthermore, despite the low concentration observed at BH4 (≤ 0.02 mg L^{-1}), the
316 extent of biodegradation for the anaerobic (control) remained the same as in the source (BH1)
317 (approximately 50 %). Therefore, a factor, other than a reduction in concentration, may be
318 influencing the extent of biodegradation at these three wells (BH4-6) (SI Figure 4).

319 The results of BH4-6 suggest sulphanilamide is being utilised as a sulphur and/or nitrogen
320 source; as opposed, or in addition to, sulphanilamide being used as a carbon source. This is
321 supported by the observation that where sulphate and/or nitrate were added to the microcosm
322 to provide a more readily available sulphur and/or nitrogen source sulphanilamide ^{14}C-
323 mineralisation was supressed (BH4-6, Figure 4, SI Figure 4). These findings are supported by
324 other research, where, sulfamethoxazole (SMX), also a member of the sulphonamide group
325 of compounds, acted as both a carbon and nitrogen source, during activated sludge
326 treatments. In particular, when utilised as a nitrogen source, biodegradation ceased when a
327 more readily available nitrogen source was present (Drillia et al., 2005). Similarly,
328 biodegradation studies of *p*-toluenesulphonamide found that, in sulphur- and nitrogen-free

329 mediums, microbes utilised *p*-toluenesulphonamide as a sulphur and nitrogen source (van
330 Haperen et al., 2001).

331 **3.3 Evaluating natural and enhanced biodegradation across the study site.**

332 Two transects, linking the sampled wells, were used to evaluate sulphanilamide
333 biodegradation down gradient of the source zone (BH1) (Figure 2) and in particular, to
334 understand the influence of an alternative carbon source (specifically, toluene). Transect 1 ran
335 from the source (BH1), 240 m down gradient in a south westerly direction, 10 mbs, through
336 B3 and onto BH6 (Figure 2). This transect is west of the toluene works and is not
337 contaminated with toluene. In contrast, Transect 2 ran from the source (BH1), 140 m down
338 gradient in a south-south-westerly direction, 10 mbs, through BH2 and onto BH5 and ran
339 close to the toluene works where the sulphanilamide plume intersects the toluene plume
340 (Figure 2).

341 **3.3.1 Transect 1 – no competing alternative carbon source.**

342 The concentration of sulphanilamide reduced by 96 % between the source (BH1, 263 mg L^{-1})
343 and BH3 (10 mg L^{-1}) (Table 1). In addition there was a shift in the redox conditions between
344 the two boreholes, from methanogenic at BH1 to Fe (III)-reduction at BH3 (Figure 4).
345 Despite these notable changes, there was no significant difference ($p \leq 0.05$) in the average
346 extent of biodegradation between the treatment variants in each of the wells (BH1, 40 - 48 %
347 and BH3, 44 - 50 %) (Figure 4), nor the same treatment variants; nitrate and sulphate,
348 between the two wells (Figure 4).

349 These results suggest sulphate and/or nitrate are not limiting factors in sulphanilamide
350 biodegradation at these two wells (BH1 and 3) and the extent of biodegradation is the same
351 for sulphanilamide concentrations $\geq$ 10 and $\leq$ 263 mg L^{-1}.

352 BH6, 330 m down gradient, from the source (BH1) (Figure 2), sulphanilamide concentrations
353 reduce to ≤ 0.02 mg L^{-1}, Fe-(III) reducing conditions remain as at BH3 (Figure 4). Two
354 distinct changes occur at BH6, compared with BH1 and 3; 1) the extent of biodegradation
355 becomes limited to ≤ 19 % (Figure 4); and, 2) the lag phase for the sulphate and nitrate
356 treatments increases from 11-14 to 40-45 days (SI Figure 4). The addition of electron
357 acceptors (sulphate and nitrate) does cause a cessation/slowing down in sulphanilamide
358 biodegradation suggesting the drivers, at BH6, are different to those up-gradient (BH1 and 3)
359 (SI Figure 4), which may also account for why the extent of biodegradation was limited to
360 ~20% compared with the 40 - 50 % observed up-gradient (BH1 and 3).

361 **3.3.2 Transect 2 – toluene present as an alternative carbon source.**

362 Sulphanilamide concentrations reduced from 263 mg L^{-1} (BH1) to 211 mg L^{-1} at well BH2
363 (80 m from the source), and to ≤ 0.02 mg L^{-1} at BH5 (140 m from the source) (Figure 2,
364 Figure 4). The observed reduction in concentration at BH5 may point towards the fringes of
365 the sulphanilamide plume, rather than the result of biological activity (Figure 1 and 2). In
366 support of this BH3, situated a similar distance from the source along transect (1) (Figure 2),
367 has a higher sulphanilamide concentration (10 mg L^{-1}) (Figure 4).

368 Although wells BH1 and BH2 have comparable sulphanilamide concentrations there was a
369 significant difference ($p < 0.05$) in the extent of biodegradation observed in the *ex-situ*
370 microcosms; with 40 ± 11 % being observed in BH1 compared with only 15 ± 10 % observed
371 in BH2 (Figure 4). No significant difference ($p \leq 0.05$) was observed between the treatment
372 variants at BH2 (Figure 4), implying nitrate and sulphate were not responsible for the
373 reduction in extent of biodegradation. However, there was a significant difference ($p \leq 0.05$)
374 in the average extent of biodegradation between the same treatment variants; nitrate and
375 sulphate, between the two wells (BH1 and 2, Figure 4).

376 This difference may be explained by the presence of another, more readily available carbon
377 source (here, specifically toluene), which could be driving a change in the biodegradation of
378 sulphanilamide. In particular, at monitoring well C (Figure 2), situated in the same area as
379 BH2, the toluene concentration is 97 mg L^{-1} (Figure 1), which could be driving the utilisation
380 of toluene as a carbon source instead of sulphanilamide. Notable are the high levels of
381 sulphate (980 mg L^{-1}), also at well C (Figure 1), some of which may be attributable to the
382 breakage of the sulphonamide bond during sulphanilamide biodegradation (van Haperen et
383 al., 2001). This elevation in sulphate at well C (Figure 1) suggests a demand for sulphur is not
384 underpinning the observed sulphanilamide biodegradation; instead toluene degradation may
385 be driving cleavage of the nitrogen from sulphanilamide.

386 At well BH5, the sulphanilamide concentration decreases to ≤0.02 mg L^{-1}, sulphate-reducing
387 conditions (-114 mV) remain as in BH2 (Figure 4). Toluene concentrations are the dominant
388 contaminant, with concentrations, reported in the same area as BH5, at 275 mg L^{-1} (well F,
389 Figure 1). The same phenomenon as seen in transect (1) at BH6 was observed, whereby,
390 adding additional nitrate to the microcosm significantly ($p \leq 0.05$) limits the extent of
391 sulphanilamide biodegradation (≤ 1 %) (Figure 4). The reduction in extent observed in the
392 nitrate treatments (BH5 and BH6, Figure 4) is not thought to be linked to low sulphanilamide
393 concentrations (≤0.02 mg L^{-1}) because nitrate additions also appeared detrimental at BH2
394 (Figure 4), where sulphanilamide concentrations were much higher (211 mg L^{-1}). These
395 results suggest sulphanilamide is being utilised as a source of nitrogen in the presence of
396 toluene.

397 Therefore, the presence of an alternative carbon source plays a role in driving the microbial
398 activity, with the results suggesting sulphanilamide biodegradation as a carbon in the source
399 area, where the extent of ^{14}C-sulphanilamide biodegradation was 40 -50 %, and a shift to
400 providing a source of sulphur and/or nitrogen in the area of the study site where an alternative

401 carbon source, i.e. toluene, existed, where the extent of ^{14}C-sulphanilamide biodegradation
402 was reduced to 15-28 %.

403 In general nitrate and sulphate augmentation did not result in significant changes to the
404 capacity of the aquifer to mineralise sulphanilamide; only where alternative carbon sources
405 were prevailing did these supplements alter sulphanilamide mineralisation. Interestingly,
406 these supplements decreased sulphanilamide degradation capacity suggesting that under *in-*
407 *situ* conditions sulphanilamide could be acting as an electron acceptor; and abating nitrogen
408 and sulphur limitations.

409 **4.0 CONCLUSION**

410 Our research provides novel insights into the mechanism that control sulphanilamide
411 biodegradation within a chalk aquifer. IRMS results identify the role of natural attenuation
412 processes in reducing sulphanilamide concentrations (from 650 to 10 mg L^{-1}) across the study
413 site, with 56 % attributable to biodegradation processes and 42 % to other natural attenuation
414 processes, such as dilution or dispersion. In addition, *ex-situ* microcosm studies showed
415 competent anaerobic biodegradation processes (40 – 50 % ^{14}C-sulphanilamide
416 mineralisation) able to degrade sulphanilamide at high (263 mg L^{-1}), moderate (10 mg L^{-1})
417 and low (0.02 mg L^{-1}) concentrations.

418 In terms of using sulphate and nitrate to enhance *in-situ* bioremediation of contaminated
419 groundwater systems, this work is of importance in a) identifying the role sulphonamide
420 compounds may play in providing a replenishable source of sulphur and/or nitrogen for
421 BTEX degraders, specifically toluene, and; b) understanding the negligible/detrimental
422 impact sulphate and/or nitrate augmentation may have on the *in-situ* biodegradation of
423 sulphonamide compounds in groundwater environments. Specifically, our results highlight

424 that under certain *in-situ* conditions sulphanilamide could be acting as a source of nitrogen

425 and sulphur.

426 Collectively, these results highlight the usefulness of both *in-situ* ($^{13}C/^{12}C$ stable isotope

427 fractionation) and *ex-situ* (^{14}C-radioisotope) monitoring tools to predict and quantify levels of

428 antibiotic biodegradation in a contaminated groundwater environment. These tools would be

429 useful to authorities who wish to evidence and quantify biodegradation of antibiotics

430 associated with natural water systems, wastewater treatment processes or agricultural run-off.

431

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Manuscript Figure Legends

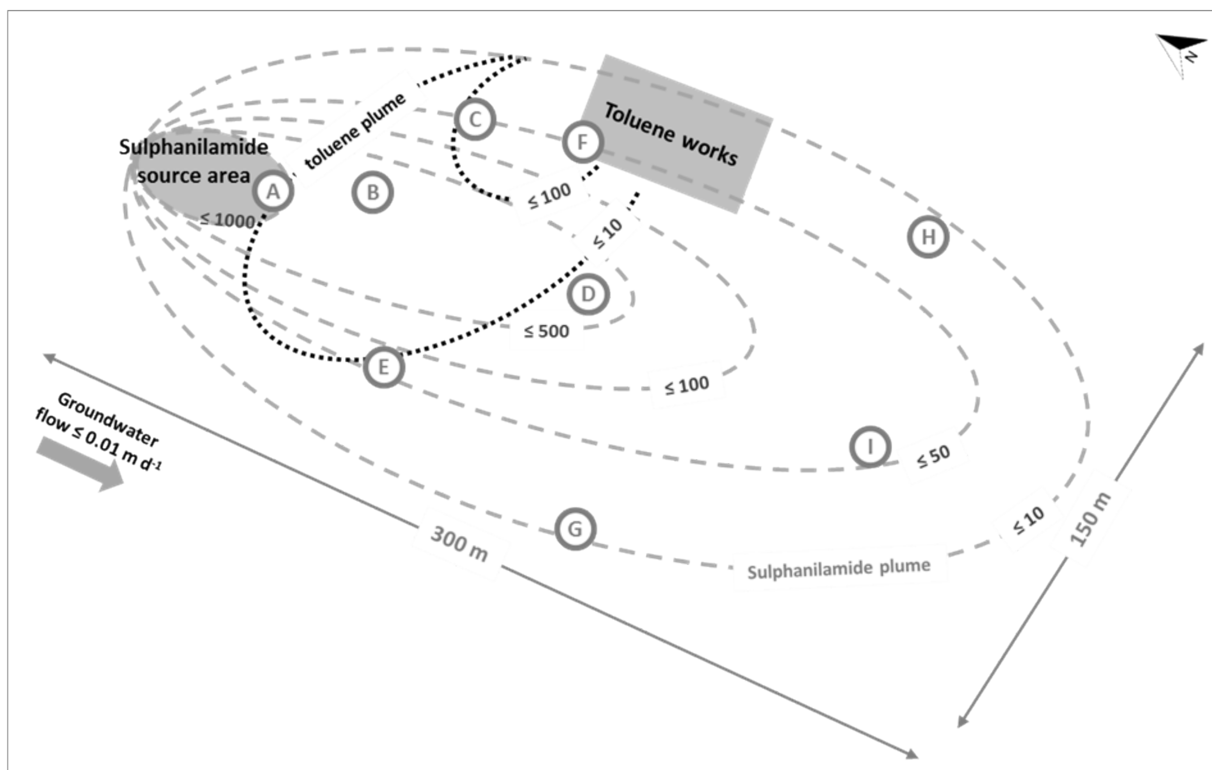
Figure 1. Schematic showing the sulphanilamide (SULPH) and toluene contaminant plumes beneath the study site and the groundwater characteristics at monitoring wells (A-I).

Figure 2. Schematic of the chemical plant showing the position of the monitoring wells (A-I), groundwater contour lines and the newly constructed boreholes (1-6), where aquifer core material and groundwater was sampled for the microcosm study.

Figure 3. Concentrations (n = 3) and $\delta^{13}C$ values (n = 3) for sulphanilamide across the site. All values are in ‰ relative to the V-PDB standard. The circles represent the depth sampled for both concentration and $\delta^{13}C$.

Figure 4. Extent of sulphanilamide biodegradation along a) Transect 1 and b) Transect 2. Upper case letters represent the significant difference ($p \leq 0.05$) between the treatment variants at each borehole whereas, lower case letters represent the significant differences ($p \leq 0.05$) between like treatment variants along the borehole transects.

621 **Manuscript Figures**



Borehole	A	B	C	D	E	F	G	H	I
SULPH ($mg\ L^{-1}$)	650	133	14	261	16	23	1	1	16
Toluene ($mg\ L^{-1}$)	7	15	97	-	15	275	-	-	-
Nitrate ($mg\ L^{-1}$)	0.6	1.5	4.1	33	17	2.5	17	-	17
Ferrous ($mg\ L^{-1}$)	2.5	0.6	14	0.1	0.3	-	0.1	9.7	-
Sulphate ($mg\ L^{-1}$)	24	30	980	140	120	140	340	53	44

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624 **Figure 1**

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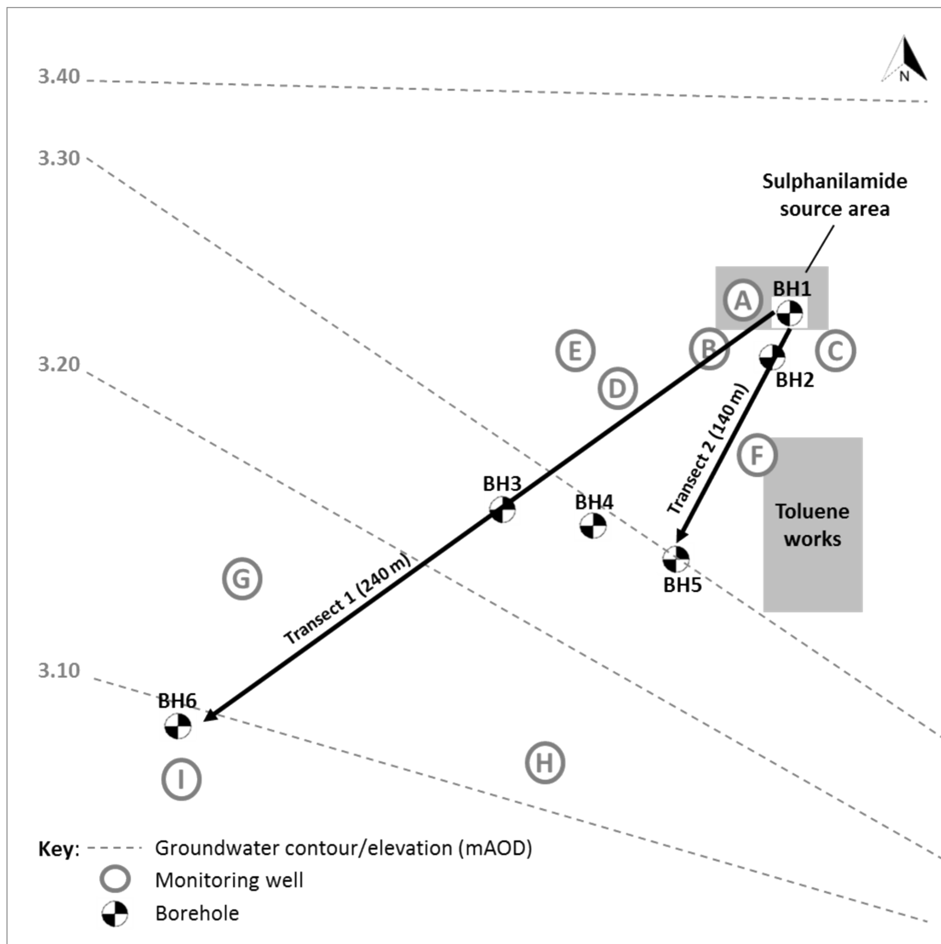

626 **Figure 2**

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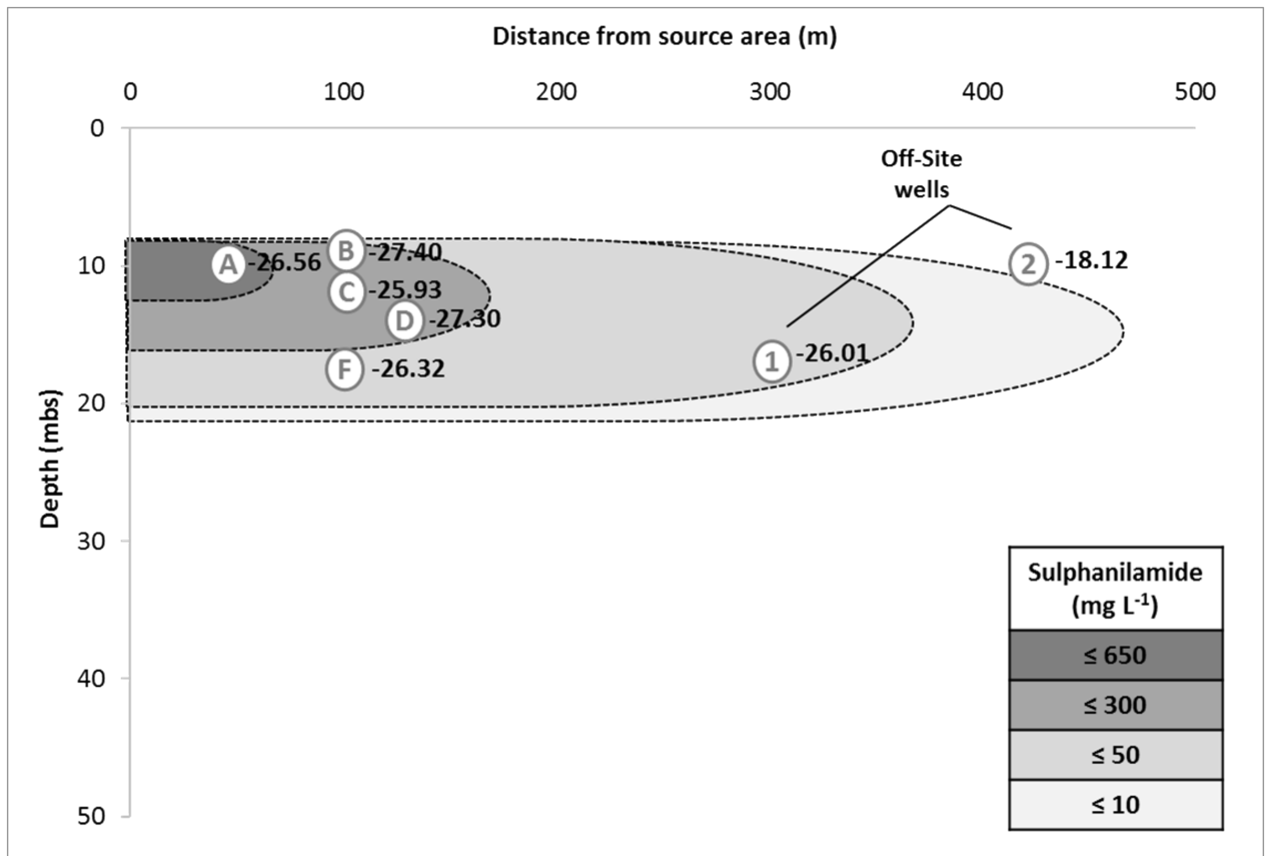

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630 **Figure 3**

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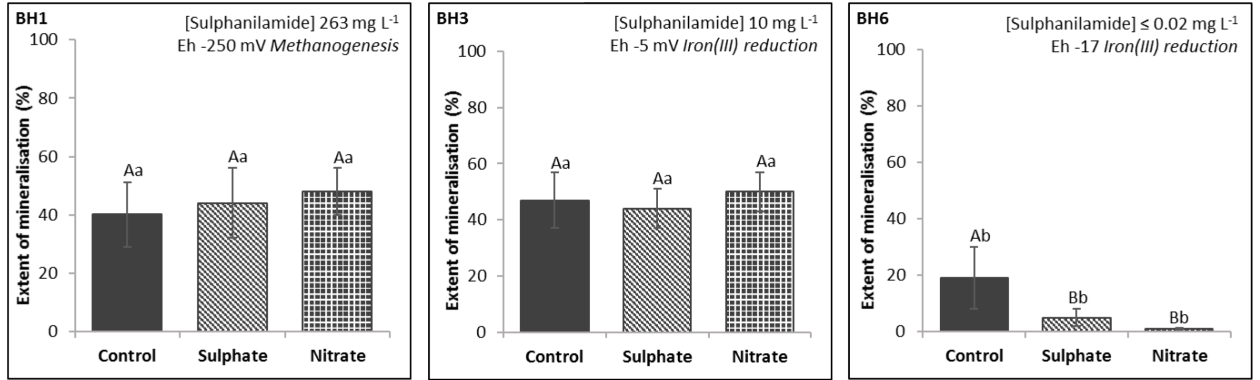

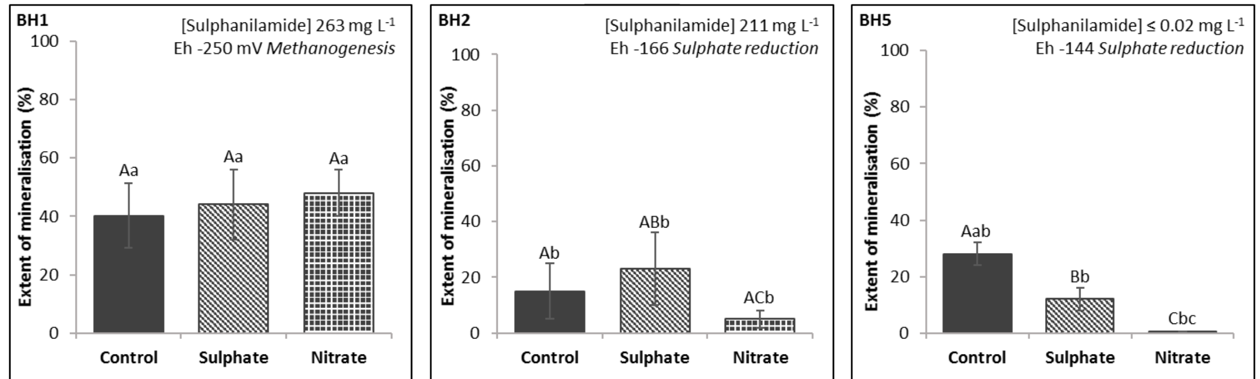

634 **Figure 4**

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644 **Supporting Information Legends**

645 **SI Box1** Calculations for the sulphate and nitrate additions to the ex-situ microcosms.

646 **SI Box2** Quantification of the degree of biodegradation for the zone between the source and a
647 monitoring point.

648 **SI Figure 1**. Redox ladder showing conditions dominant across 77 monitoring wells at the
649 field study site. Prevailing redox conditions are those samples situated above the data line for
650 each redox potential.

651 **SI Figure 2**. Stable isotope fractionation linearity test: mass of sulphanilamide (mg) vs. peak
652 amplitude of mass 44 (V). The linear regression of the curve gave an R^2 of 0.992, suggesting
653 good linearity between signal strength and increasing amount of analyte.

654 **SI Figure 3**. Plot of peak amplitude of mass 44(V) vs. δ13Csulphanilamide (‰). Plot of
655 mean (solid black line) and standard deviation (hatched lines) show at low signal values (< 5
656 V) the IRMS may report more enriched δ13C values and at high signal values (>10 V) more
657 depleted δ13C values. Therefore, the linear equation (SI Figure 2) was used to ascertain a
658 minimum quantity of sulphanilamide (0.2 mg at approximately 6 V).

659 **SI Figure 4.** Mineralisaiton of ^{14}C-sulphanilamide observed in the anaerobic control (●),
660 nitrate-augmented (☐) and sulphate-augmented (△) microcosm over a period of 84 days.

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667 **Supporting Information Figures**

Calculations for sulphate and nitrate additions to *ex-situ* microcosms:

Calculations were based on stoichiometric equations (Eq. 4 and 5) for the complete denitrification and/or sulphate reduction of 263 mg L^{-1} sulphanilamide (i.e. the sulphanilamide concentration at BH1 (Figure 4) to ensure sufficient quantities of the electron acceptors were present to facilitate this mode of biodegradation.

Denitrification:

$$C_6H_8N_2O_2S + 6NO_3^- + 12H^+ + 6e^- \rightarrow 6CO_2 + 6H_2O + 4N_2 + SO_2 \quad [4]$$

Based on equation [4], the reduction of 6 mole of nitrate to nitrogen consumes 1 mole of sulphanilamide.

Sulphate reduction:

$$1.5C_6H_8N_2O_2S + 5.5SO_4^{2-} + 28H^+ + 17e^- \rightarrow 9CO_2 + 7H_2S + 7H_2O + N_2 \quad [5]$$

Based on equation [5], the reduction of 5.5 mole of sulphate to hydrogen sulphide consumes 1.5 mole of sulphanilamide.

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669 **SI Box1**

Quantification of the degree of biodegradation for the zone between the source and a monitoring point:

Step 1:

Fractionation factor (α):

$$\alpha = R_a/R_b$$

R is the stable isotope ratio ($^{13}C/^{12}C$) of the compound and the subscripts a and b represent the compound in the source zone, versus a down gradient monitoring point.

Step 2:

$$f = \left[R_b/R_a \right]^{1/(\alpha_{ab}-1)}$$

Fraction remaining (f):

R_b is the isotope ratio for downgradient monitoring well and R_a the isotope ratio for source or up gradient monitoring point.

Step 3:

$$B = (1-f)*100$$

The amount of biodegradation as a percentage of material originally present (B)

Step 1: (α = 1.47) Step 2: (f = 0.44) Step 3: (B = 56 %)

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671 **SI Box2**

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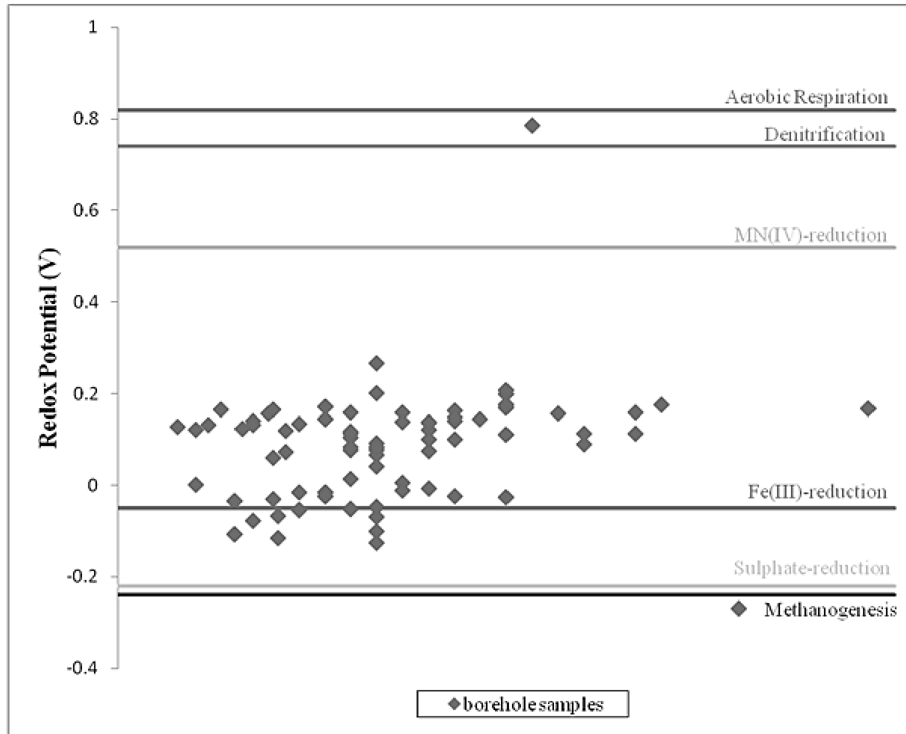

673 **SI Figure 1**

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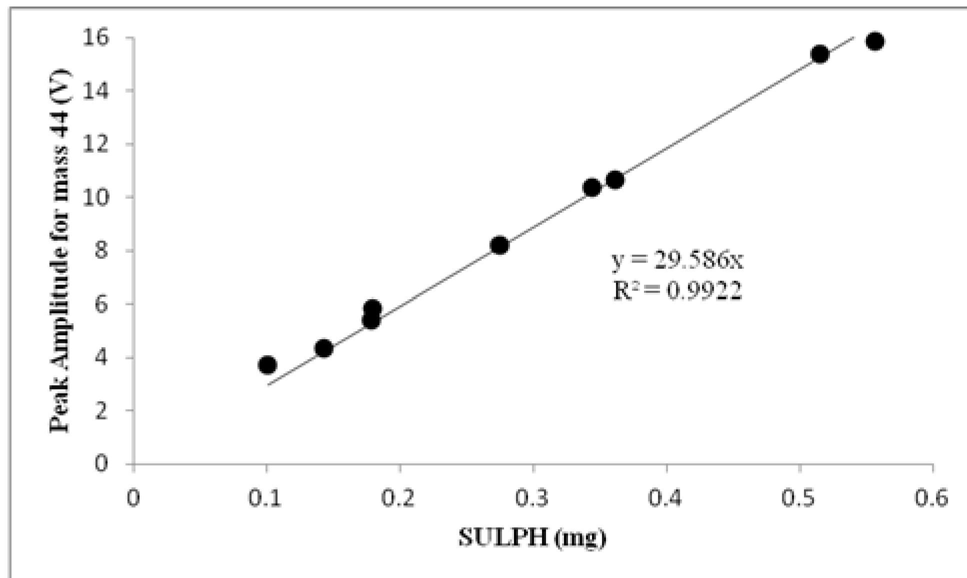

675 **SI Figure 2**

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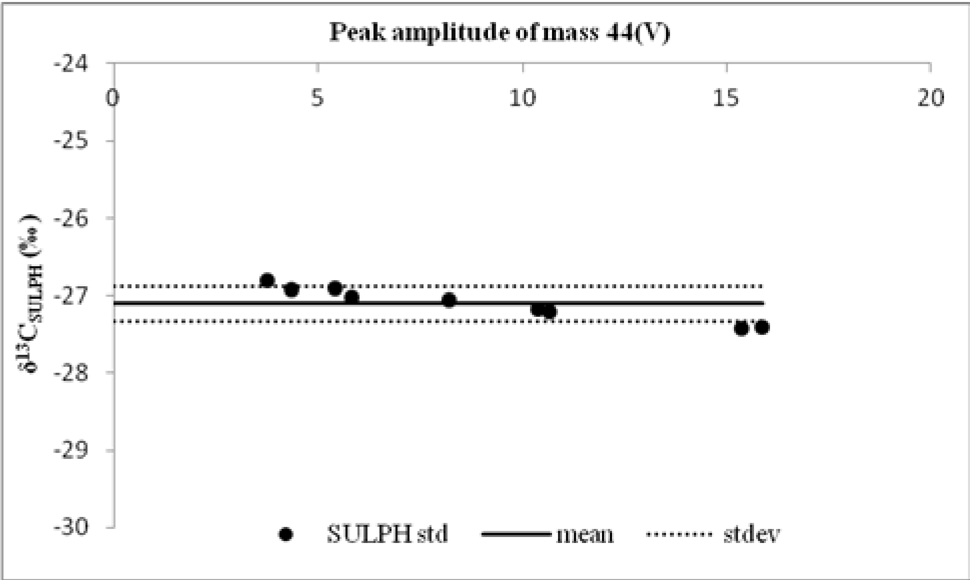

677 **SI Figure 3**

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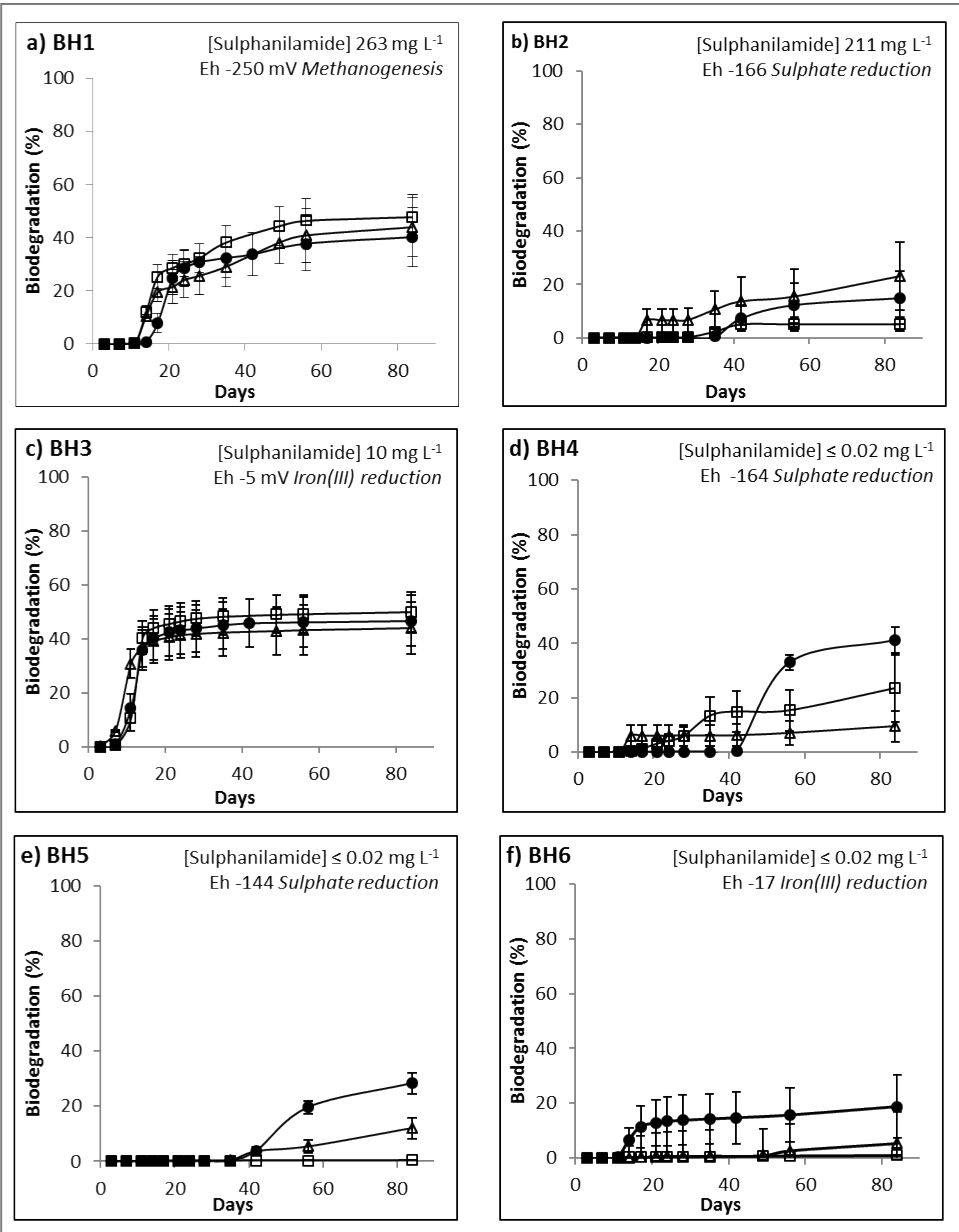

679 **SI Figure 4**

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