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1 **Title of article:** Microbial changes linked to the accelerated degradation of the herbicide atrazine in a range of
2 temperate soils

3

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24 **Abstract**

25 Accelerated degradation is the increased breakdown of a pesticide upon its repeated application, which has
26 consequences for the environmental fate of pesticides. The herbicide atrazine was repeatedly applied to soils
27 previously untreated with *s*-triazines for >5 years. A single application of atrazine, at an agriculturally relevant
28 concentration, was sufficient to induce its rapid dissipation. Soils with a range of physico-chemical properties
29 and agricultural histories, showed similar degradation kinetics, with the half-life of atrazine decreasing from an
30 average of 25 days after the first application to <2 days after the second. A mathematical model was developed
31 to fit the atrazine degrading kinetics, which incorporated the exponential growth of atrazine-degrading
32 organisms. Despite the similar rates of degradation, the repertoire of atrazine degrading genes varied between
33 soils. Only a small portion of the bacterial community had the capacity for atrazine degradation. Overall the
34 microbial community was not significantly affected by atrazine treatment. One soil, characterised by low pH,
35 did not exhibit accelerated degradation and atrazine degrading genes were not detected. Neutralisation of this
36 soil restored accelerated degradation and the atrazine-degrading genes became detectable. This illustrates the
37 potential for accelerated degradation to manifest when conditions become favourable. Additionally, the
38 occurrence of accelerated degradation under agriculturally relevant concentrations supports the consideration of
39 the phenomena in environmental risk assessments.

40

41 **Keywords:** microbial communities, atrazine, risk assessment, fate modelling, soil pH, adaptation

42 **Introduction**

43 In the EU registration of a new plant protection product requires a risk assessment which includes evaluation of
44 its environmental fate (EC, 2009). This is strongly affected by degradation processes (Katayama et al. 2010)
45 which are often mediated by microorganisms (Dodge et al. 2012; Howell et al. 2014). Upon the repeated
46 application of a pesticide or its analogue microbial communities are able to adapt and degrade the pesticide a
47 faster rate, referred to as accelerated degradation (Racke 1990). Accelerated degradation has been shown to
48 occur for a broad range of pesticides classes (Arbeli and Fuetes 2007), although it is not currently considered in
49 the EU registration studies. For example to determine the transformation of a chemical in soil, such studies
50 specify that the soils used for the test must not been treated with the substance or its analogues for four years
51 (OECD, 2002), however it has been shown that the capacity for pesticide degradation can be maintained for up
52 to 10 years (Cheyns et al. 2012).

53

54 Accelerated degradation has the potential to reduce pesticide persistence in the environment (Shaner et al.
55 2007), but also to deplete the efficacy of a pesticide (Krutz et al. 2008). This may contribute to pressure on
56 pesticide use in the light of the limited number of active substances (Chapman, 2014) and increased pest
57 resistance (Heap 2016). The phenomenon is dependent on the microbial community, however the specific
58 changes that occur in the microbial community between pesticide applications and lead to the faster rate of
59 degradation, are poorly understood (Arbeli and Fuetes 2007; Itoh et al. 2014).

60

61 The herbicide atrazine (1-Chloro-3-ethylamino-5-isohpropylamino-2, 4, 6-triazine) was selected as a model
62 pesticide to determine the changes that occurred in the microbial community during accelerated degradation.
63 Atrazine was selected as it is highly effective and extensively used around the world (Syngenta 2016), although
64 banned in the EU since 2003 (EC 2015). Additionally, its microbial degrading pathway is well characterised
65 (Udikovic-Kolic et al. 2012), see Online Resource 1, which enabled the microbial capacity for the degradation
66 of atrazine to be tracked.

67

68 Previous studies that have examined the accelerated degradation of atrazine used high concentrations of atrazine
69 to isolate highly tolerant microorganisms for bioremediation (Cai et al. 2003; Wang et al. 2014), often around
70 agrochemical factories, (Udikovic-Kolic 2008; Udikovic-Kolic 2010) or atrazine treated agricultural fields
71 (Zablutowicz et al. 2007). This may have biased microbial changes in favour of the fastest growing and most

72 readily culturable members of the community (Dunbar et al. 1997). We used soils with no documented history
73 of *s*-triazine application and applied atrazine at an agriculturally relevant rate to gain an insight into the potential
74 for accelerated degradation in soils that have previously been un-treated, or at least have not recently had
75 concerted exposure to atrazine. We then tracked the microbial changes linked to accelerated degradation as it
76 manifested. Pyrosequencing of the 16S rRNA bacterial gene was adopted to enable the community to be
77 analysed more comprehensively compared to fingerprinting techniques such as fatty acid methyl ester (FAME)
78 which had been done previously (Zablotowicz et al. 2007), and which have been shown to lack resolution (Bent
79 et al. 2007). We concentrated on the bacterial portion of the community, as they have been shown to be mainly
80 responsible for accelerated degradation (Walker 1993).

81

82 A broad range of soil properties have been associated with influencing accelerated degradation from plant
83 exudates (Piutti et al. 2002) to moisture content (Schroll et al. 2006) and carbon availability in the soil (Popov et
84 al. 2005, Ngigi et al. 2013). Specifically for atrazine soil pH has shown to affect degradation (Houot et al. 2000).
85 The effect of pH has been examined previously, but not linked to detection of the atrazine degrading genes or
86 the manifestation of accelerated degradation.

87

88 Currently there is no attempt to account for accelerated degradation in the models used for pesticide dissipation
89 for regulatory purposes, despite the potentially enormous influence of adaptive, biological pesticide degradation
90 on both product effectiveness and residual concentration in soils. To address this, we developed a growth-linked
91 model based on the accumulation of pesticide degrading microorganisms to fit the kinetics of atrazine
92 disappearance and to facilitate incorporation of accelerated degradation into environmental risk assessments.

93

94 **2. Materials and methods**

95 Three major groups of experiments were conducted in this study; the first to examine the microbial changes
96 associated with accelerated degradation, the second to determine the effect of soil properties on accelerated
97 degradation and the third to explore the effect of pH on accelerated degradation. All three groups of experiments
98 monitored atrazine dissipation to determine the capacity of the soils for accelerated degradation and the presence
99 of the atrazine degrading genes to track accelerated degradation. The soils used for each experiment are detailed
100 in Table 1 and the sampling regime is detailed in online resource 2.

101 **2.1. Soil collection**

102 Nine soils which had no documented history of being treated with *s*-triazines were collected from 4 UK farms,
103 with different physical and chemical properties; Cotril (C): 54° 8' 2.832" N, 0° 58' 36.098" W; Mount (M): 54°
104 5' 36.218" N, 1° 1' 38.770" W; Grange (GR): 54° 6' 10.703" N, 0° 50' 9.082" W and Ganthorpe (G): 54° 7'
105 27.026" N, 0° 56' 48.793" W. The latitude and longitude for each farm were determined from postcodes inputted
106 into <http://www.latlong.net/> (LatLong, 2012-2014) and converted into coordinates using
107 <http://www.sunearthtools.com> (SunEarthTools.com, 2009-2016).

108

109 From each farm two soils with different management histories were removed. One soil had been out of
110 agricultural practice for over 5 years, and was referred to as set-aside (S) while the other soil, that had been
111 under continuous agricultural practice, including pesticide treatment for over 5 years (Online Resource 3), was
112 referred to as the agricultural (A) soil. The set aside soils had slightly different management histories as follows;
113 CS and GRS were grassland, whilst MS was a buffer strip and GS was fallow.

114

115 All soils were collected in 2013 apart from the Ganthorpe soils, which were first collected in 2012, and soil from
116 the agricultural site was resampled in 2013, to see if the repertoire of pesticide degrading genes had changed. In
117 addition the soil for the pH study was collected from Grange in 2014 (Table 1). At each field site, the debris was
118 removed from the soil surface and then ~ 10 kg of soil, from the top 10 cm was transferred into bags and kept at
119 4 °C for up to 12 hours prior to processing.

120 **2.2. Soil characterisation**

121 Soil pH was measured in H₂O in 1:2.5 w/v suspensions (Avery and Bascomb 1974) using a pH probe. The pH
122 probe was calibrated and 10 g of soil added into a 50 mL polyethene beaker with 25 mL of distilled water,
123 stirred and left to stand for 10 min. The pH probe was then introduced, and recorded when stable. The moisture
124 content of each soil upon sampling was determined in grams water per gram of oven dried soil (105 °C), shown
125 in Table 1, while the maximum water holding capacity was determined using the Avery and Bascomb method
126 (Avery and Bascomb 1974).

127 Total organic carbon was measured using the Walkley and Black method (Walkley and Black 1934), total
128 nitrogen using the AOAC method (AOAC 1990) and the soil textural class USDA using the Black method
129 (Black 1965), conducted by Natural Resource Management (NRM) Ltd., Berkshire, UK.

130 **2.3. Microcosm construction**

131 Soils were processed in accordance with OECD 307 guidelines for analysis of chemical transformation in soils
132 (OECD 2002) as follows: soils were air-dried then sieved to 2 mm, moisture adjusted to 40 – 60 % of the
133 MWHC and were maintained at $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ in the dark. The soils GA_2012 and GS_2012 consisted of 12
134 treated subsamples and 4 control samples -the extra treated samples enabled an assessment of the variation in
135 atrazine recoveries between replicates to be made. For the soils collected in 2013 and 2014, 8 subsamples of 400
136 g (on a dry weight basis) were transferred into glass amber jars secured with foam bungs. For each soil 4 jars
137 were treated with atrazine and 4 jars were untreated controls.

138

139 As a sterile control, 4 x 10 g of GRA soil replicates were autoclaved at $121\text{ }^{\circ}\text{C}$ for 15 min., treated with atrazine
140 as above, and sampled in quadruplicate at day 0, 1, 3, 7 and 14 days.

141 **2.4. pH adjustment**

142 The soils GRA_pH and GRS_pH had their pH altered according to the method applied by Nicol et al. (2008).
143 GRS (pH 5.4) had 2 mg g^{-1} of lime ($\text{Ca}(\text{OH})_2$) added to maintain $\sim\text{pH } 7$ (Online Resource 4), and was referred
144 to as GRS amended (GRSa). The GRA_pH soil (pH 6.2) had 8 mg g^{-1} of aluminium sulphate ($\text{Al}_2(\text{SO}_4)_3$) added
145 and was maintained at $\sim\text{pH } 4$ throughout the study (Online Resource 4), and was referred to as GRA amended
146 (GRAa). Soil pH was monitored in a non-atrazine treated control pot weekly for each soil and each amendment
147 added as required, followed by moisture adjustments.

148 **2.5. Atrazine application**

149 Atrazine (PESTANAL, Sigma Aldrich) was applied to 4 amber jars per soil type (12 for the GA_2012 and
150 GS_2012). Due to its low water solubility atrazine was dissolved in methanol and added to 5 g of 1 mm silica
151 sand. The methanol was left to evaporate and the sand mixed into the soil samples. Atrazine was applied at a
152 final agriculturally relevant concentration of $6\text{ }\mu\text{g g}^{-1}$ of dry soil (Tomlin 2009). The four control samples per
153 soil, had silica sand with evaporated methanol added. Atrazine was applied in this way three times (twice for the

154 pH study) over an interval of 60 days for the first application and 28 days between the second and third
155 application.

156 **2.6. Atrazine extraction & detection**

157 Samples were removed for analysis 0, 1, 3, 7, 14, 28, 45 and 60 days (day 45 samples were not removed for the
158 GA_2012 and GS_2012) after the first atrazine application and 0, 1, 3, 7, 14, 28 days after the second and third
159 application.

160

161 Atrazine was extracted from 1 g (dry weight basis) soil sub-samples by homogenisation with 20 mL of methanol
162 and shaken on a side-side shaker at 230 rpm for 30 min. Following centrifugation (2500 rpm for 5 min), 10 mL
163 of the supernatant was filtered (cellulose acetate 0.45 μm) and 2 mL of the filtrate concentrated to dryness under
164 a flow of nitrogen at 35 °C. The residue was then re-suspended in 200 μL of methanol:water (50:50) using a
165 vortex mixer. Extracts were transferred to HPLC vials and stored at -20 °C prior to analysis.

166

167 The concentration of atrazine was determined on the Agilent 1100 series and 1200 series UV module HPLC
168 using a methanol:water mobile phase (50:50) at 1 mL min^{-1} , injection volume of 20 μL , separated on a C18
169 column with UV detection of atrazine at 222 nm after ~ 8.3 min. The estimated limit of detection (LOD) based
170 on the lowest calibration standard was 0.02 $\mu\text{g mL}^{-1}$. The chromatograms were manually integrated using the
171 Chemstation software in order to estimate peak areas, which were then converted into concentrations from
172 calibration curves. Calibration curves were prepared by producing atrazine standards in methanol:water (50:50)
173 at six concentrations from 0.02 $\mu\text{g mL}^{-1}$ to 5.0 $\mu\text{g mL}^{-1}$. Plots of atrazine concentration vs. peak area were
174 constructed and linear regression used for determining the atrazine concentration in the samples.

175 **2.7. Modelling of atrazine dissipation**

176 Modelling of atrazine dissipation over the three applications and in the pH and sterile control samples, was
177 conducted according to the recommendations of the forum for the co-ordination of pesticide fate models and
178 their use (FOCUS 2006) to obtain estimated values of the dissipation time 50 (DT_{50}). The percentage recoveries
179 of atrazine from the theoretical amount applied were modelled using the KinGUIi software v2. Initially the data
180 was optimised to fit the single first order (SFO) model. The visual fit of the data, χ^2 value and spread of the
181 residuals were used to determine if a biphasic model would be a better suited to the data, dependent on whether
182 10 % of the initial measured concentration had been reached (FOCUS 2006). A biphasic model was only fitted

183 to the data if compared to SFO the biphasic model resulted in an improved visual fit, low Chi squared (χ^2)
184 estimate (< 15 %) and the model parameters passed the t-test (FOCUS 2006).

185

186 To take into account the impact of accelerated degradation on atrazine removal, an alternative approach to
187 modelling the atrazine degradation data was developed, referred to as the ‘growth linked model’. This involved
188 accounting for the increase in a community of biological atrazine degraders over time during the incubation of
189 soils, as accelerated degradation has been associated with an increase in degrader abundance (Bending et al.
190 2001). The model consisted of two rates of degradation: (i) a first order exponential decay rate (chemical), and
191 (ii) a biological decay rate, dependent hyperbolically on atrazine concentration. The difference in atrazine
192 concentration [Atr] between two time points (time t, and time t+n) was calculated computationally as follows;

$$[Atr](t+n) = [Atr]_t - \left(Y \times n \times k_1 \times e^{-k_1 t} \right) - \left(N \times n \times V \times [Atr] \div \left([Atr] + K_s \right) \right)$$

193
194

195 For the exponential term, Y is the percentage of atrazine that is available for degradation, n is the length of the
196 time-step (typically set at 0.01 days) used in the modelling, k_1 is the exponential atrazine decay rate. For the
197 hyperbolic term, N is the size of the atrazine degrader community, V is the maximum rate of atrazine removal,
198 K_s is the Michaelis constant representing the concentration of atrazine that gives half the maximum rate of
199 hyperbolic atrazine degradation. The size of the atrazine degrader community (N) changes over time as the
200 community of atrazine degrading organisms grows (as the soil community adapts to atrazine being available). N
201 is calculated as a number between 0 and 100, by the following equation:

$$N = N_0 e^{\mu t}$$

203 where μ is the exponential growth rate of atrazine degraders and N_0 is the initial size of the atrazine degrading
204 community capable of growth. N is limited to a maximum arbitrary size of 100.

205

206 The model was implemented using a custom-made script written in Python, and the parameters determined
207 based on qualitative fit to the data.

208 **2.8. Sorption**

209 To investigate the effect of soil pH on sorption of atrazine, batch sorption experiments were conducted as
210 described in OECD 106 (OECD 2000). Prior to the batch sorption experiment atrazine was determined to be
211 stable in 0.01 M CaCl_2 for at least 24 hours. Sorption of the atrazine was estimated for the GRS_pH and

212 GRA_{pH} soils and following alteration of their pH GRSa and GRAa, using the standard batch sorption method
213 detailed in OECD 106 (OECD 2000).

214

215 Five grams of each soil, in duplicate tubes were pre-equilibrated with 22.5 mL of 0.01M CaCl₂ by shaking at
216 200 rpm on a side to side shaker overnight. Atrazine stock solutions of 0.2 µg mL⁻¹, 5.0 µg mL⁻¹ and 20.0 µg
217 mL⁻¹ were added in either 2.5 mL or 1 mL volumes to obtain theoretical concentrations of 0.02 µg mL⁻¹, 0.08 µg
218 mL⁻¹, 0.20 µg mL⁻¹, 0.80 µg mL⁻¹ and 2.0 µg mL⁻¹. Additional 0.01 M CaCl₂ was added to ensure all tubes had
219 25 mL of 0.01 M CaCl₂, to achieve a 1:5 soil to solution ratio. After atrazine addition soil suspensions were
220 returned to the shaker for 24 hrs to reach pseudo-equilibrium. The samples were then centrifuged at 3000 rpm
221 for 5 min and the supernatants then filtered (0.2 µm PTFE membrane filters) into 2 mL HPLC vials which were
222 stored at 4 °C prior to analysis.

223

224 The final solute concentration of atrazine in solution after adsorption (C_{aq}) was determined from the HPLC of
225 the supernatant, assuming that all atrazine removed from solution, has been adsorbed. The concentration sorbed
226 to soil (C_s) was calculated as below;

$$227 \quad C_s = K_f \times C_e^{1/n}$$

228 Values for the Freundlich adsorption coefficient (K_f) and the regression constant (n) for the Freundlich
229 adsorption equation were obtained using a solver in Excel by selecting values that minimised the sum of the
230 least squares between measured and modelled values. C_{aq} values were then plotted against C_s to examine the
231 change in sorption as a function of concentration.

232 **2.9. Measuring ATP**

233 Total Adenosine Triphosphate (ATP) was extracted from 7 days after each application in triplicate from
234 GA₂₀₁₂ and GS₂₀₁₂ to monitor the total microbial activity. The Celsis Beverage Kit™ (Brussels, Belgium)
235 was used to measure ATP and the positive control kit (Celsis) used to check the functioning of the Celsis
236 Cellscan M201B luminometer. For the positive control sample 1 g of sterilised soil was mixed with 10 mL of
237 nuclease-free water (Severn biotech Ltd, Worcestershire, UK) and 100 µL of *E. coli* (NCTC 9703) cell
238 suspension, while a blank cuvette was used as a negative control. The ATP content of 1 g (dry weight) of soil
239 was used for all reactions and 10 mL of nuclease-free water (Severn biotech Ltd, Worcestershire, UK) was
240 added and samples were shaken and processed using the Celsis Beverage Kit. Initially the variability of ATP

241 (measured in relative light units) was measured in 3 subsamples of the same atrazine un-treated soil sample, two
242 aliquots of the soil sludge were then recorded, the variation between the subsamples was determined not to be
243 significant, using a Student's t-test; all $p > 0.20$, (Online Resource 5). One gram sub-samples from atrazine
244 treated pots were monitored in triplicate 7 days after each application.

245 **2.10. DNA extraction & PCR**

246 Total community DNA was isolated from the atrazine treated and control soils across the three applications of
247 atrazine (two applications for the pH study). Approximately 5 g of soil per sample were homogenised in an
248 automatic shaker (Merris Engineering Ltd, Galway, UK) for 2 min., with 10 mL of CTAB (Cetyl Trimethyl
249 Ammonium Bromide) buffer (120mM sodium phosphate buffer pH 8, 2 % CTAB, 1.5 M NaCl), 0.3 mL of
250 antifoam B (Sigma-Aldrich, Dorset, UK) and 10 metal ball bearings (10 mm diameter). The supernatant was
251 removed and centrifuged at 2000 g for 2 min., and vortexed with 250 μ L of Food Buffer B (Promega, Madison,
252 USA) until it appeared milky. This was followed by addition of 750 μ L of Precipitation Buffer (Promega) which
253 was vortexed and centrifuged at 13000 g for 10 min. The extracted DNA was then purified using the Promega
254 wizard food kit, in conjunction with the Kingfisher™ mL system (Thermo Fisher Scientific Inc., Massachusetts,
255 USA) with a magnetic particle processor using the "gDNAnew" programme. The programme was as follows:
256 750 μ L of the cleared sample was mixed with 600 μ L of isopropanol with 50 μ L of the magnesil beads
257 (Promega) for 10 min and the genomic DNA bound to the magnetic particles, transferred to 1 mL of lysis buffer
258 B (Promega) for 2 min, followed by 4 washes in 1 mL of 70 % ethanol for 2 minutes each, followed by 5 min.,
259 of heating at 65 °C and final elution in 200 μ l of TE buffer (pH 8). The purity of extracted DNA was determined
260 using the nanodrop (ND 1000 3.3) system (Thermo Fisher Scientific Inc), and frozen at -20 °C in TE buffer (10
261 mM Tris, 1 mM EDTA, pH 8).

262 **2.11. Detection & sequencing of atrazine degrading genes**

263 Samples from different time points across the three applications of atrazine (2 applications for the pH study)
264 were checked for atrazine degrading genes (Online Resource 1). The PCR mix consisted of 1x KAPA HiFi
265 fidelity buffer (Kapa Biosystems, Woburn, MA, USA), 0.3 μ M of dNTPs, 0.3 μ M of each primer (Table 2), 1 U
266 μ L⁻¹ KAPA HiFi polymerase and nuclease-free water (Severn biotech Ltd, Worcestershire, UK) to reach 25 μ L
267 final volume. The PCR followed these thermal cycling conditions: initial denaturation at 95 °C for 5 min, and 30
268 cycles of denaturation at 98 °C for 30 sec, annealing for 15 sec (at the specified temperature in Table 2) and 15
269 sec elongation at 72 °C, followed by a final extension of 5 min at 72 °C (Bio-Rad Laboratories, Inc, USA).

270 Products were visualised on a 2 % agarose gel, containing 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide for DNA binding.
271 Bands of the expected size were gel extracted using the Qiagen gel purification kit (Qiagen, Hilden, Germany)
272 and quantified using nanodrop v3.3. Amplicons at concentrations of 4-10 $\text{ng } \mu\text{L}^{-1}$ were re-suspended in nuclease-
273 free water (Severn Biotech Ltd, Worcestershire, UK) and 0.3 μM of the forward primer added and directly
274 sequenced using the Applied Biosystems instrument 3130XL (CA, USA). DNA sequences were analysed using
275 the Sequence Scanner 1.0 software and similarity to previously sequenced genes was determined from the NCBI
276 using the nucleotide BLAST tool (Altschul et al. 1990).

277

278 Genomic DNA that did not produce a detectable atrazine degrading gene product was tested for the effect of
279 inhibitors by adding 2 μL aliquots of the potentially “inhibitory” genomic DNA to a working PCR using
280 undiluted, and diluted DNA extracts (1:10 and 1:100), failure to produce a PCR product in an initially working
281 PCR following addition of the “inhibitory” genomic DNA would indicate that the gene may not be absent in that
282 gDNA sample, but its amplification may have been prevented by inhibition. However in this study no inhibition
283 of the PCR was evident.

284 **2.12. Real Time PCR**

285 Relative quantification of *trzN* was performed by Q-PCR to estimate the proportion of the community in the
286 soils GA_2012 and GS_2012 containing the atrazine degrading gene. The *trzN* gene was selected as the gene of
287 interest it was the most commonly identified atrazine degrading gene in this study, in-line with previous studies
288 (Arbeli and Fuetes 2010).

289

290 The 16S ribosomal RNA gene was selected as a normalisation gene, due to its presence in all bacteria, although
291 different copy numbers are found in some species (Acinas et al. 2004). Quantification of *trzN* could then be
292 compared between different samples, despite differences in the number of bacteria and or concentration of
293 gDNA template. Primers for the gene targets (TrzN_Q_F & R, 16S_Q_F & R, the latter from Yang et al. (2002)
294 were selected using the Primer Express® Software for Real-Time PCR version 3.0 (Applied Biosystems) and
295 synthesised by Eurofins MWG Operon. Primers are listed in Table 2. The Q-PCR method was adapted from
296 Udikovic-Kolic et al. (2010) and was performed on an Applied Biosystems StepOne™ instrument using SYBR
297 Green® for detection in 20 μL reactions. Each reaction consisted of 10 μL of Power SYBR® Green Mix 2 x
298 (Applied Biosystems), 6.2 μL Nuclease-free dH_2O (Ambion®), 0.4 μL of each primer (5 μM each) and 3 μL of

299 gDNA. Reactions were run in 96-Well Optical Reaction Plates (Applied Biosystems) for relative quantification,
300 according to the manufacturer's instructions.

301

302 Thermal cycling conditions were as follows; hold at 95 °C for 10 min, 40 cycles at 95 °C for 15 seconds and 60
303 °C for 1 min. The final step was added initially to produce a melt curve, starting from 60 °C to 95 °C to ensure
304 that a single product was produced. Each sample was run in triplicate per target, to obtain average Ct (cycle
305 threshold) values.

306

307 Standard curves of *trzN* and *16S rRNA* were constructed using purified PCR products for these genes amplified
308 from a soil sample that had been treated with atrazine three times. The PCR amplicons were purified using the
309 Qiagen gel purification kit and quantified based on absorbance at 260 nm using Nanodrop v3.3. Amplicons were
310 required to have a 260/280 ratio between 1.8 and 2.0. For relative quantification standard curves were produced
311 by serially diluting the amplicons tenfold, four times for *trzN* and five times for *16S rRNA*. The standard curves
312 *16S rRNA* and *trzN* are shown in Online Resource 6 and 7, respectively. Plots of log DNA concentration vs. Ct
313 value were constructed and the linear regression line used for determining the gene concentration in the sample.
314 The percentage of the bacterial community containing *trzN* was then calculated using the following formula;

$$315 \frac{trzN \text{ gene concentration}}{16S rRNA \text{ gene concentration}} \times \frac{\text{length of } 16S \text{ gene product (bp)}}{\text{length of } trzN \text{ gene product (bp)}} \times 100$$

316

317 Significant differences in the portion of the community containing *trzN* between treated and control samples, for
318 each soil was determined by unpaired t-tests.

319 **2.13. Community analysis**

320 To determine the bacteria present in the GA_2012 and GS_2012 soils, the V3-V5 fragment of the 16S rRNA
321 gene was amplified by the polymerase chain reaction (PCR) and pyrosequenced using primers previously tested
322 by Klindworth et al. (2013). PCR primers were adapted to 454 amplicon sequencing, for which a M13 adapter
323 (bold and underlined) was attached to the target forward primer Bakt_341F (5'-
324 **CACGACGTTGTAAAACGAC**CCTACGGGNGGCWGCAG-3'). To aid multiplexing different samples,
325 different barcodes were included using the M13 adapter. Sequence adapter A (bold) was followed by the 454
326 amplicon sequencing specific 4-mer amplification key (italics) followed by a 10-mer sequence (NNNN) barcode
327 and M13 (bold and underlined) (5'-

328 **CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNN****CACGACGTTGTAACGAC** -3'). An
329 overview of barcode sequences used can be found in Online Resource 8. The 25-mer Lib-L specific sequence
330 adapter B was followed by the reverse template specific primer sequence Bakt_805R (*italics*) (5'
331 CCTATCCCCTGTGTGCCTTGGCAGTCGACTACHVGGGTATCTAATCC -3').
332
333 The PCR mix consisted of 1x KAPA HiFi fidelity buffer (Kapa Biosystems, Woburn, MA, USA), 0.3 μM of
334 dNTPs, 0.3 μM of each primer, 1 U μL⁻¹ KAPA HiFi polymerase, 0.3 μM M13 adapter and nuclease-free water
335 (Severn Biotech Ltd, Worcestershire, UK) to reach 25 μL final volume. The PCR followed these thermal
336 cycling conditions: initial denaturation at 95 °C for 5 min, and 30 cycles of denaturation at 98 °C for 30 sec,
337 annealing for 15 sec at 55.3 °C and 15 sec elongation at 72 °C, followed by a final extension of 5 min at 72 °C
338 using the Bio-Rad C1000 (Bio-Rad Laboratories, Inc, USA). Products were visualised on a 1 % agarose gel,
339 containing 0.5 μg mL⁻¹ ethidium bromide for DNA binding. Band intensity of 16S rRNA gene amplicons of the
340 correct size (469 bp) was used to estimate quantity for pooling. Subsequently, pooled amplicons were run on a
341 3.5 % gel to separate out small fragments, which were excised and extracted using the Qiagen Gel purification
342 kit according to the manufacturer's instructions. This concentrated pooled sample was heated for 5 min at 95 °C
343 and snap cooled on ice for 2 min. This was followed by a second gel electrophoresis on a 2 % gel of the
344 concentrated pooled amplicons. The excised band of correct size was extracted using the Qiagen kit. The purity
345 and concentration was determined using Nanodrop (ND-1000 3.3) and DNA fragment pattern assessed using a
346 DNA 1000 Chip on the Agilent Bioanalyser, 2100 series (Agilent Technologies Inc, CA, USA).
347
348 The sequences of partial 16S rRNA genes were obtained using a Roche GS-FLX 454 pyrosequencer (Roche,
349 Mannheim, Germany) using picotiter sequencing plates and sequenced as advised by the manufacturer for
350 amplicon sequencing. Samples were processed through the QIIME (quantitative insights into microbial ecology)
351 pipeline (Caporaso et al. 2010b). Initially samples were filtered by quality (-M4; maximum number of primer
352 mismatches, -s 30; minimum average quality score allowed in read) and were split by their barcode sequence.
353 The number of sequences present before and after quality filtering is shown in Online Resource 9. Clustering
354 into operational taxonomic units (OTUs) was then performed using UCLUST at the 97 % similarity level,
355 indicative of species level (Edgar 2010). The most abundant sequences were chosen as being representative of a
356 cluster and aligned with the PYNAST method (Caporaso et al. 2010a). The OTU table generated was then
357 rarefied to 2910 sequences per sample, to avoid bias. OTUs present in 1 or 2 samples were removed from the

358 rarefied OTU table (L6) and this table was then transformed by square root and a Bray Curtis resemblance
359 matrix was constructed in PRIMER6 (Primer-E Ltd, Lutton, UK).

360

361 The Bray-Curtis matrix was clustered using hierarchical-clustering with group-average linkage to produce a
362 dendrogram representing the scaled similarity between samples. Nonmetric multidimensional scaling (nMDS)
363 plots were used to provide a visual representation of the similarities between bacterial communities, based on
364 the Bray Curtis similarity index. On the nMDS plots the clustering of data points was highlighted by overlaying
365 ellipses based on the clustering. The significance of bacterial community clustering was quantified using
366 analysis of similarity (ANOSIM) which is an analogue to the standard univariate one-way ANOVA (analysis of
367 variance) designed for ecological data. ANOSIM generates an R statistic that indicates the separation between
368 groups where an R of 1 indicates complete separation and R of 0 indicates there is no separation (Clarke et al.
369 2006).

370

371 To test the variation in the bacterial community explained by each variable PERMANOVA was calculated using
372 the Adonis function in the R package vegan (Oksanen et al. 2013). A matrix of variables (atrazine, soil and
373 incubation time) versus samples was constructed that corresponded to the relative abundance of each OTU in
374 each sample. The test statistic and associated *p* value was calculated using 999 random permutations on the
375 basis of Bray Curtis distances. A *p* value was calculated using the classical F distribution approximation. The
376 significance level to reject the null hypothesis was set a priori to 0.05. Results were visualised using R (version
377 3.2.1) R Core Team, 2015.

378 **2.14. Principal Component Analysis**

379 To explore the variation between the 9 soils, principal component analysis (PCA) was conducted in PRIMER v6
380 (Clarke et al. 2006), based on different soil properties. A draftsman plot showed the data points were equally
381 spread, therefore multivariate normality was assumed. Each variable was normalised (subtraction of the mean
382 and dividing by the standard deviation) to provide comparable, dimensionless scales for a correlation based
383 PCA. The PCA was composed of five principal components and the eigenvalues, eigenvectors and principal
384 component scores were used to determine the soil properties that best explained the variation between sites.

385 **2.15. Accession numbers**

386 The 16 S rRNA amplicons have been deposited in the SRA with the accession number; SRP066748
387 (PRJNA304340).

388

389 **3. Results**

390 ***3.1. Soils of different physico-chemical properties exhibited a similar pattern of accelerated degradation.***

391 Eight out of the nine soils untreated by *s*-triazines for at least 5 years and banned since 2003 (EC, 2015)
392 demonstrated accelerated degradation, evident by their faster rate of dissipation following a second application
393 of atrazine (Fig.1).

394

395 Using the European regulatory FOCUS guidance (FOCUS, 2006), data from the first application of atrazine
396 fitted single first order (SFO) kinetics adequately for all soils, with good visual fits and X^2 error values of < 15
397 % (Table 3). After the second and third applications several soils had poor SFO visual fits missing several
398 points, and this was not improved by fitting the data to biphasic kinetics. Initially four soils; CS, CA, GA and
399 GRS, had estimated DT_{50} values of greater than 30 days and the remaining soils had DT_{50} values of greater than
400 19 days. By the second application all soils, apart from GRS, had DT_{50} values of 3 days or less (Table 3). After
401 the third atrazine application all soils apart from GRS had DT_{50} values of less than 2 days (Table 3). The
402 parameters used for the SFO fits are in the Online Resource 10.

403

404 All eight soils exhibiting accelerated degradation showed a 7 – 32 fold reduction in their DT_{50} values from the
405 first to the second application of atrazine, whereas the DT_{50} of GRS was 48.6 days after the third application
406 (Table 3), with the amount of atrazine appearing to accumulate after each application (Fig. 1).

407

408 It is evident that the vast majority of these soils are exhibiting an accelerated rate of atrazine degradation within
409 60 days of applying atrazine. Using a sterile control, it was shown that sterile soil displayed a DT_{50} of 107.7
410 days compared to 20.4 days in a matched non-sterile soil (Online Resource 11). This confirmed in-line with
411 previous studies, that accelerated degradation of atrazine is microbially driven (Zablotowicz et al. 2007).

412 However, the modelling approaches applied above do not take into account the kinetics of microbial growth or
413 microbial adaptation over multiple applications. In fact, the SFO kinetics applied to the first application of
414 atrazine fails to capture the rate of atrazine decay, which clearly begins to accelerate between 28 and 45 days or

415 45 and 60 days for most soils. This is illustrated in the GRA soil in which time points at 28 days and 60 days
416 deviate significantly from the exponential fit (Fig. 2). Similar poor fits are also observed during the degradation
417 of the first atrazine application in the other soils (Online Resource 12 - 18).

418

419 Therefore we addressed this by developing a model that included an initial rate of exponential atrazine
420 degradation and an activity following hyperbolic, Monod-type kinetics, consistent with a microbially mediated
421 biodegradation. This latter activity was dependent on the size of a microbial community, capable of atrazine
422 utilization. This community was modelled to increase exponentially up to a limit, as would be expected for a
423 microbial community *in vivo*. This more sophisticated approach was able to capture the change in atrazine
424 degradation for each of the degrading soils and accounted for the variation in rate of degradation between
425 atrazine additions. For example the GRA data fitted using the microbial-growth model fits the data much better
426 than the individual exponential fits for each atrazine application (Fig. 2). The growth-linked model enabled a
427 single set of parameters to be used to fit all the atrazine degradation kinetics, rather than using separate
428 parameters for each atrazine addition.

429

430 Overall, the growth-linked model gave a superior fit for the data from all 8 soils exhibiting accelerated
431 degradation (Fig. 2 and Online Resource 12 - 18). The parameter that varied the most widely was the initial
432 number of atrazine degraders in the community (Online Resource 19), which varied by 3 orders of magnitude
433 (from 0.00005 to 0.03). The other fitted variables had values in the ranges: k_1 from 0.06 – 0.18, Y from 30 – 65
434 %, μ from 0.155 to 0.225 day^{-1} , V from 0.6 – 0.7, $K_s = 75$ in all cases. The K_s value of 75 is a percentage of the
435 initial applied atrazine concentration ($6 \mu\text{g.L}^{-1}$), i.e $K_s = 20.9 \mu\text{M}$. This result from modelling is remarkable
436 given the atrazine degrading enzyme TrzN has been measured to have $K_m = 20 \mu\text{M}$ (Shapir et al. 2005) and 25
437 μM (Topp et al. 2000).

438 ***3.2. The atrazine degrading genes were detectable in soils exhibiting accelerated degradation***

439 To determine if the soils exhibiting accelerated degradation carried the atrazine degrading genes and the extent
440 to which the repertoire of genes varied between soils, DNA from 3, 14 and 28 days after each application of
441 atrazine was tested. All soils that exhibited accelerated degradation contained at least *trzN* while none of the
442 atrazine degrading genes were detectable in GRS (Table 4) which did not exhibit accelerated degradation (Fig.
443 1).

444 The presence of the atrazine degrading genes (Online Resource 1) was determined for all nine soils. Soil MS
445 exhibited accelerated degradation (Fig. 1), but only *trzN* could be detected (Table 4) and was characterised by its
446 high clay content of 22 % and a low C:N ratio of 9.6 (Table 1).

447

448 In five of the soils that exhibited accelerated degradation (GA_2012, MA, CA, CS and GRA) three atrazine
449 degrading genes were detected (Table 4). All these soils had a pH of greater than 6.3 (Table 1). *AtzA* and *atzB*
450 were detected in six of the eight soils showing accelerated degradation (Table 4), while *atzC* was only detected
451 in GA. The gene *trzD* of the lower atrazine degrading pathway, which is more tightly regulated and less
452 commonly identified (Udikovic-Kolic et al. 2012), was only detected in GA_2012 and GS_2012 (Table 4). It is
453 also notable that resampling of the GA_2012 soil in 2013 (GA) led to a different repertoire of atrazine degrading
454 genes being detectable, with the detection of *atzA* and *atzC* which were below the limit of detection in 2012,
455 while *trzD* was below the limit of detection in 2013. All of the atrazine degrading genes detected had sequences
456 which were 100 % identical to those characterised previously (Online Resource 20).

457 **3.3. Atrazine treatment does not have a gross impact on the overall microbial community**

458 To determine whether the increase in atrazine degradation was due to an overall increase in microbial activity in
459 soils, soil ATP content was measured following repeated applications of atrazine. There was no significant
460 change in ATP concentration in soils over time (Online Resource 21) indicating that a significant increase in the
461 microbial community is unlikely to explain the increased degradation of atrazine, and that a more likely reason
462 would be proliferation of specific microorganisms containing the atrazine degrading genes.

463

464 It is clear that there is a significantly greater proportion of the bacterial community containing *trzN* after the
465 second and third application of atrazine in the agricultural soil, and after the second application in the set aside
466 soil ($p < 0.05$) (Fig. 3). It is also evident that only a small proportion (< 0.5 %) of the bacterial community
467 contained *trzN*.

468

469 Non-metric multidimensional scaling was conducted using ANOSIM to observe clustering of soil samples
470 according to various variables (Fig. 4). The low abundance of *trzN* relative to the overall number of bacteria
471 present is consistent with the absence of significant clustering between samples based on atrazine treatment
472 (ANOSIM; R: 0.08, p : 0.25). This is also consistent with analysis by PERMANOVA showing that the variation

473 in the bacterial communities was most affiliated with their duration of incubation ($R^2 = 0.23$, $p = 0.0001$) and soil
474 type ($R^2 = 0.17$, $p = 0.0002$) rather than atrazine treatment ($R^2 = 0.08$, $p = 0.09$).

475 **3.4 GRS was most strongly associated with pH**

476 Atrazine was found to be at least 19 times more persistent in GRS compared to any other soil, following the
477 second and third application of atrazine (Table 3). The soil properties that distinguished this soil from the others
478 that exhibited accelerated degradation were investigated. The 9 soils used in this study had been naïve to *s*-
479 triazines for 5 or more years (Online Resource 3) and the DT_{50} values in the set aside soils, that had not had
480 pesticides applied were very similar to those seen in the agricultural soils (Table 3). Therefore it was unlikely
481 that exposure to pesticides other than *s*-triazines affected accelerated degradation.

482

483 PCA was used to determine the soil physical and chemical properties (Table 1) that explained the variation
484 between soils and those most correlated with the GRS soil, which may have affected its ability to mediate
485 accelerated degradation.

486

487 It is clear that the 7 soils are scattered and do not cluster dependent on the farm they originated from or whether
488 they were collected from set aside or agricultural sites (Fig. 5). PC1 explained 68 % of the variation and PC2, 20
489 %, with most of the remainder explained by PC3 (8.4 %), together explaining 96.3 % of the variation in soil
490 properties (Online Resource 22). It can be inferred that PC1 is roughly an equal weighted combination of most
491 of the soil properties including texture (sand, silt and clay), nutrients (Organic Carbon and Total Nitrogen) and
492 moisture parameters (moisture content (MC) and maximum water holding capacity (MWHC)) with the greatest
493 contribution from sand and organic carbon contents (Online Resource 23).

494

495 PC2 is mainly explained by pH, although there is some contribution from carbon to nitrogen ratio (C:N) and
496 moisture content too, while PC3 is mainly explained by C:N ratio (Online Resource 23). Based on the principal
497 component scores, it was clear that the variation between the agricultural soils; CA, GA and MA were mainly
498 dependent on PC1 (Online Resource 24). Specifically, CA had the highest sand content, while GA and MA had
499 the lowest and highest organic carbon contents, respectively (Table 1). GRA was an outlier from the other soils
500 mainly affiliated with PC3 (Online Resource 24) and explained by having the highest C:N ratio (Table 1),
501 whereas MS and GRS were strongly affiliated with PC2, due to them having the highest and lowest pH
502 respectively.

503 **3.5. Soil pH affected the capacity of the soils to mediate accelerated degradation of atrazine**

504 The GRS soil was the only one not to exhibit accelerated degradation, and was notably the soil with the lowest
505 pH. To determine whether the lack of accelerated degradation was linked to the low pH, and whether the genetic
506 potential for atrazine degradation is retained in this low pH soil, the pH of the GR soils was experimentally
507 altered. GRA which had a pH of 6.2 and had exhibited accelerated degradation (Fig. 1) was acidified and
508 maintained at ~ pH 4 to suppress accelerated degradation, while GRS which had a pH of 5.4 and did not exhibit
509 accelerated degradation (Fig. 1) was neutralised and maintained at ~ pH 7, to determine whether accelerated
510 degradation could be induced.

511

512 After the second application of atrazine, the concentration of atrazine was below the limit of detection at day 14
513 and day 28 in GRSa and at day 28 in GRA, showing how rapid accelerated degradation of atrazine had occurred
514 in the amended GRS sample. Accelerated degradation was only observed in GRSa and GRA (Fig. 6) which both
515 had a pH greater than pH 6.2. The DT₅₀ values of these soils were less than 1.6 days after the second application
516 of atrazine although the soils with pH < 5.4.; GRS and GRAa, exhibited DT₅₀ values that were longer than 21
517 days (Table 3), indicating that atrazine will be more persistent in these soils. In addition to its low pH GRS is
518 also affiliated with high contents of clay, moisture and organic carbon (Table 1).

519

520 Sorption was shown to increase with atrazine concentration (Online Resource 25) and was slightly higher in the
521 acidic soils GRAa and GRS than the neutral soils GRA and GRSa but these differences were small, and
522 insufficient to explain the >7 fold difference in atrazine dissipation rates between these treatments (Table 3 and
523 Fig. 6).

524

525 To see if the change in soil pH modified detection of the atrazine degrading genes in the treated soil PCRs of the
526 atrazine chlorohydrolase genes; *atzA* and *trzN* were conducted. Neither *atzA* nor *trzN* could be detected after
527 multiple applications of atrazine in the acidic soils GRS and GRAa. Contrastingly, both *atzA* and *trzN* were
528 detectable in soils at pH > 6.2 (GRA and GRSa) after 2 applications of atrazine, indicating that the genes are
529 present in both GRA and GRS, but only proliferate to become detectable at near neutral pH.

530

531 **4. Discussion**

532 ***4.1. Accelerated degradation manifested in soils with no history of s-triazine use after one application***

533 Eight soils transitioned to an accelerated rate of dissipation within 60 days (Fig. 1) demonstrating that most soils
534 tested had the capacity for the accelerated degradation of atrazine. The soils had no *s*-triazine history
535 documented and atrazine has been banned since 2003 (EC 2015). Previous studies have shown that accelerated
536 degradation was evident in soils that had received two annual applications of atrazine in the field and the rates of
537 mineralisation in these soils were much lower (Zablotowicz et al. 2007; Houot et al. 2000), compared to the very
538 rapid rate of atrazine degradation seen in this study, which could be explained by the shorter interval between
539 applications. In addition most previous studies that have demonstrated accelerated degradation have used soils
540 with long histories of atrazine applications (Zablotowicz et al. 2007). Although spray drift of simazine, which
541 has been applied more widely in the UK (D. Garthwaite: pesticide usage survey; personal communication),
542 cannot be ruled out as a source of low level *s*-triazine availability stimulating these soils, this does not detract
543 from the finding that accelerated degradation of atrazine occurs in soils that have no recorded history of
544 deliberate *s*-triazine application. Such high potential for the accelerated degradation of atrazine could mean that
545 soils with history of *s*-triazine use over 4 years ago may be ineffective, although this will need to be investigated
546 in a field and the extent to which this applies to other pesticides explored. Additionally Orlikowska et al. (2015)
547 detected atrazine in marine waters which they presumed to have reached the sea by run off from agricultural
548 land. An interesting extension to this project would be to investigate the movement of atrazine residues and
549 potentially atrazine degrading organisms, via artificial drainage or run-off.

550

551 ***4.2. Accelerated degradation was associated with the atrazine degrading genes***

552 To examine the microbial changes linked to accelerated degradation, general activity, microbial community
553 structure and the capacity for degradation were monitored. No increase in general biological activity was linked
554 to accelerated degradation (Online Resource 21) in agreement with other studies De Andrea et al. (2013) saw no
555 correlation between dehydrogenase activity and glyphosate mineralisation, whilst Udikovic-Kolic et al. (2011)
556 demonstrated major shifts in microbial communities treated with atrazine. In this study we found there was no
557 significant change in the microbial community in response to atrazine treatment (Fig. 4). This probably relates
558 to the levels of atrazine employed. In the current study, atrazine was used at the recommended agricultural level,
559 whereas the previous analysis concerned a highly contaminated pesticide site (Udikovic-Kolic et al. 2011).

560 Accelerated degradation was associated with detection of the atrazine degrading genes of the hydrolytic
561 pathway, which were detected upon the increased rate of dissipation of atrazine. There is an alternative atrazine
562 degrading pathway; the oxidative-hydrolytic pathway for atrazine dissipation which degrades atrazine via the
563 production of deethylatrazine (DEA) and deisopropylatrazine (DIA) (Giardina et al. 1982). However the
564 oxidative-hydrolytic pathway has mainly been associated with non-adapted soils that have a slow rate of
565 degradation (Fournier et al. 1997). In addition hydroxyatrazine has been shown to be the dominant metabolite in
566 atrazine adapted soils (Kruz et al. 2010) and in atrazine-mineralising cultures (Yanze-Kontchou and Gschwind
567 1994; Mandelbaum et al. 1995; De Souza et al. 1998). PCR was used in an attempt to identify the *thcBCD* genes
568 involved in the oxidative-hydrolytic pathway (Shao and Behki 1996), but were not detected in this study.

569

570 ***4.3. Accelerated degradation of atrazine was associated with a small portion of the microbial community***

571 The repertoire of atrazine degrading genes was variable between soils, and was shown to vary after one year
572 between subsequent samples of the GA soil, possibly due to changes in the exact location the soil was removed
573 (Bending et al. 2001). The high degree of variability in apparent gene content between soils (Table 4), yet the
574 similar atrazine degradation rate following adaptation (Fig. 1), suggests that limitations in the method may also
575 have been an issue. This is also reflected in the relatively low percentage of the microbial community composed
576 of atrazine degraders (estimated to be 0.5 %), (Fig. 3). However the low proportion of atrazine degraders may
577 also have been affected by the low concentration of atrazine available for them to degrade. As Baelum et al.
578 (2006) saw that microbial growth was more pronounced when the bacterial community was supplied with high
579 pesticide concentrations compared to low concentrations. However Udikovic-Kolic et al. (2010) found that only
580 1 - 4 % of their atrazine community contained the atrazine degrading genes, even when the atrazine
581 concentrations were 100 x greater than applied in this study and Sneigowski et al. (2012) showed that only 0.5
582 % v/v of a pesticide degradation inoculum was required for maximum pesticide degradation.

583

584 ***4.4. A new microbial growth linked model enables accelerated degradation to be considered in environmental*** 585 ***risk assessments***

586 To enable accelerated degradation to be considered in the risk assessment we modelled our data in-line with the
587 current regulatory approach for modelling pesticide dissipation to a microbial based approach. Fitting the
588 disappearance of atrazine using the standard FOCUS Guidance (FOCUS 2006) used in EU pesticide regulation,

589 showed a drastic reduction in DT_{50} between the first and second application of atrazine (Table 3). However,
590 SFO kinetics that are the preferred option to derive regulatory degradation endpoints, failed to capture the
591 change in atrazine concentration during the incubation with the first application of atrazine (Fig. 2, Online
592 Resource 12 - 18). To remedy this, we generated a model that took into account the exponential growth of
593 atrazine degraders. This microbial growth model gave better fits to all data sets with much tighter residuals (Fig.
594 2, Online Resource 12 - 18) than existing accepted methods, which do not consider microbial growth and
595 adaptation between applications as only one application is modelled at a time. Models that build in growth of
596 bacteria during an adaptation phase may have widespread applicability in studies of adaptation to pesticides, but
597 also other microbially catalysed processes in natural and agricultural environments, such as monitoring the
598 numbers of degraders involved in bioremediation (Fuetes et al. 2016).

599
600 The parameters obtained from these fits indicated that a key variable between soils is the initial number of
601 atrazine degraders prior to atrazine addition. This varied by up to 3 orders of magnitude between the soils
602 (Online Resource 19), but the final rates of atrazine degradation after adaptation were remarkably stable. It is
603 worth noting that the highest initial numbers of atrazine degrading organisms used to fit the data were over 3000
604 times less than after adaptation, which may help explain why atrazine degrading genes were initially
605 undetectable in this study.

606

607 ***4.5. The repertoire of the atrazine degrading genes differed between the soils exhibiting accelerated*** 608 ***degradation***

609 The final objective of this study was to examine the impact of soil properties on atrazine degradation and its
610 genetic potential. It was shown that all soils that exhibited accelerated degradation contained *trzN*. *TrzN* has
611 been identified as being more prevalent than the alternative atrazine chlorohyrolase; *AtzA* (Arbeli and Fuetes
612 2010) attributed to its catalytic superiority and wider substrate against a range of triazine herbicides (Shapir et
613 al. 2007; Shapir et al. 2005). In addition *atzA* and *atzB* were detected in six of the eight soils showing
614 accelerated degradation. *AtzB* enables nitrogen to be obtained from atrazine (Seffernick et al. 2007). Although
615 *AtzA* is a homologue of *TrzN* and having both enzymes is not required to degrade atrazine, it has been
616 suggested that communities with both may be more tolerant to new environmental conditions (Udikovic-Kolic
617 et al. 2012). *AtzC* could only be detected in GA, which provides isopropylamine released from the s-triazine ring

618 which can be used as carbon, nitrogen and/or energy sources for bacterial growth (Strong et al. 2002). In
619 agreement with other studies few genes of the lower atrazine degrading pathway were detected (Udikovic-Kolic,
620 et al. 2012) possibly due to atrazine not being the primary substrate for bacteria in most soils that are not
621 contaminated with high concentrations of atrazine or alternatively that other unidentified pathways can catalyse
622 these metabolic reactions.

623 The atrazine degrading genes have been identified to be highly conserved in pure cultures and the environment
624 (De Souza et al. 1998a; Sagarkar et al. 2013) and were identical in this study to those identified previously (De
625 Souza et al. 1998a; Sagarkar et al. 2013; Mulbry et al. 2002).

626

627 ***4.6. The capacity for accelerated degradation in an acidic soil was restored upon neutralisation***

628 GRS was the only soil not to exhibit accelerated degradation. Instead, atrazine accumulated after each
629 application and the atrazine degrading genes were not detected in this soil. This soil had the highest carbon
630 content, which has previously been associated with reducing atrazine mineralisation by providing an alternative
631 carbon source to atrazine and / or increasing sorption (Popov et al. 2005; Ngigi et al. 2013). Although the carbon
632 content may have contributed to the accelerated degradation of GRS the PCA analysis showed that a low pH
633 was the defining feature of GRS (Figure 5). A low pH (<6) has previously been associated with inhibiting
634 accelerated degradation of atrazine (Houot et al. 2000; Mueller et al. 2010) and a similar observation was made
635 by Singh et al. (2003) who saw for the insecticide Chloropyrifos that upon transfer of a pesticide degrader to an
636 acidic soil the identified degrader was no longer detectable. Here we showed that the biological potential for
637 atrazine degradation was present in low pH, non-degrading soils (GRS) and was expressed following
638 amendment of these soils to neutral conditions, which allowed recovery of accelerated degradation, an activity
639 that was correlated with the detection of known atrazine degrading genes. This is the first study to demonstrate
640 that the low pH soils retain the genetic potential for atrazine biodegradation. The vast majority of European
641 agricultural soils have a soil pH of 5-7, therefore the abundance of the atz/trz genes could mean a broad range of
642 soils have the potential for accelerated degradation.

643

644 **Conclusions**

645 The accelerated degradation of atrazine occurred in soils that had not been treated with the pesticide or its
646 homologues, at agriculturally relevant levels, and was related to the detection of the atrazine degrading genes.

647 The atrazine degrading genes were found in all of the soils tested, but were shown to vary dependent on soil
648 properties, demonstrating that the ability to degrade a pesticide is widespread and can be retained for many
649 years. We suggest that accelerated degradation should be explicitly considered in the risk assessment process to
650 gain a more realistic view of pesticide efficacy and fate.

651

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656

657 **References**

658 Acinas SG, Marcelino, LA, Klepac-Ceraj V, Polz MF (2004) Divergence and redundancy of 16S rRNA
659 sequences in genomes with multiple rrn operons. *J Bacteriol* 186:2629-2635

660

661 Altschul, SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic Local Alignment Search Tool. *J Mol Biol*
662 215:403-410

663

664 AOAC (1990) AOAC: Official methods of analysis. 15th Ed. Association of Official Analytical Chemists, Inc.
665 Arlington, Virginia 22201, USA

666

667 Arbeli Z, Fuentes CL (2007) Accelerated biodegradation of pesticides: an overview of the phenomenon, its basis
668 and possible solutions; and a discussion on the tropical dimension. *Crop Prot* 26:1733-1746

669

670 Arbeli Z, Fuentes CL (2010) Prevalence of the gene *trzN* and biogeographic patterns among atrazine-degrading
671 bacteria isolated from 13 Colombian agricultural soils. *Fems Microbiol Ecol* 73:611-623

672

673 Avery BW, Bascomb CL (eds.) (1974) Soil Survey Laboratory Methods. Technical Monograph No. 6, Adlard &
674 Son Ltd, Dorking, UK

675

676 Bælum J, Henriksen T, Hansen HCB, Jacobsen CS (2006) Degradation of 4-Chloro-2 Methylphenoxyacetic
677 Acid in Top- and Subsoil Is Quantitatively Linked to the Class III *tfdA* Gene. *Appl Environ Microb* 72:1476-
678 1486

679

680 Bending, GD, Shaw E, Walker A (2001) Spatial heterogeneity in the metabolism and dynamics of isoproturon
681 degrading microbial communities in soil. *Biol Fert Soils* 33:484-489

682

683 Bent S J, Pierson J D, Forney LJ (2007) Measuring species richness based on microbial community fingerprints:
684 The emperor has no clothes. *Appl Environ Microb* 73:2399-2399

685

686 Black C. (1965) Particle fractionation and particle-size analysis. American Society of Agronomy Madison, pp
687 550-551
688

689 Cai B, Han Y, Liu B, Ren Y, Jiang S (2003) Isolation and characterization of an atrazine-degrading bacterium
690 from industrial wastewater in China. *Lett Appl Microbiol* 36:272-276
691

692 Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R (2010a). PyNAST: a flexible
693 tool for aligning sequences to a template alignment. *Bioinformatics* 26:266-267
694

695 Caporaso JG, Kuczynski J, Stombaugh J J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pêa AG, Goodrich
696 JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge
697 BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunencko T, Zaneveld J,
698 Knight R (2010b) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7:335-
699 336
700

701 Chapman P (2014) Is the regulatory regime for the registration of plant protection products in the EU
702 potentially compromising food security? *Food Energ Secur* 3:1-6
703

704 Cheyns K, Martin-Laurent F, Bru D, Aamand J, Vanhaecke L, Diels J, Merckx R, Smolders E, Springael D
705 (2012) Long-term dynamics of the atrazine mineralization potential in surface and subsurface soil in an
706 agricultural field as a response to atrazine applications. *Chemosphere* 86:1028-1034
707

708 Clarke KR, Clarke RK, Gorley RN (2006) Primer V6: User Manual - Tutorial: Plymouth Marine Laboratory
709

710 De Andréa MM, Peres TB, Luchini LC, Bazarin S, Papini S, Matallo MB, Savoy VLT (2013) Influence of
711 repeated applications of glyphosate on its persistence and soil bioactivity. *Pesqui Agropecu Bras* 38:1329-1335
712

713 De Souza ML, Seffernick J, Martinez B, Sadowsky MJ, Wackett LP (1998) The Atrazine Catabolism Genes
714 *atzABC* Are Widespread and Highly Conserved. *J Bacteriol* 180:1951-1954
715

716 Devers M, Soulas G, Martin-Laurent F (2004) Real-time reverse transcription PCR analysis of expression of
717 atrazine catabolism genes in two bacterial strains isolated from soil. *J Microbiol Meth* 56:3-15
718

719 Dodge AG, Wackett LP, Sadowsky MJ (2012) Plasmid Localization and Organization of Melamine Degradation
720 Genes in *Rhodococcus* sp Strain Mel. *Appl Environ Microb* 78:1397-1403.
721

722 Dunbar J, White S, Forney L (1997) Genetic Diversity through the Looking Glass: Effect of Enrichment Bias.
723 *Appl Environ Microb* 63:1326-1331
724

725 EC (2009) Regulation (EC) No 1107/2009 of the European Parliament and the council of 21 October 2009
726 concerning the placing of plant protection products on the market. Edited by E. Union: Official Journal of the
727 European Union
728

729 EC (2015) European Commission. EU Pesticides Database: Atrazine.
730 <http://ec.europa.eu/food/plant/pesticides/eu-pesticides>
731 [database/public/?event=activesubstance.detail&language=EN&selectedID=972](http://ec.europa.eu/food/plant/pesticides/eu-pesticides/database/public/?event=activesubstance.detail&language=EN&selectedID=972). Accessed 31 October 2015
732

733 Edgar, R. C (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26: 2460-246
734

735 FOCUS (2006) Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental
736 Fate Studies on Pesticides in EU Registration. Report of the FOCUS Work Group on Degradation Kinetics. In
737 EC Document Reference Sanco/10058/2005, pp 1-434
738

739 Fournier J, Soulas G, Parekh N (1997) Main microbial mechanisms of pesticide degradation in soils. In:
740 Tarradellas J (ed.) *Soil ecotoxicology*. Lewis Publishers CRC, New York, pp 85-116
741

742 Fruchey I, Shapir N, Sadowsky MJ, Wackett LP (2003) On the origins of cyanuric acid hydrolase: Purification,
743 substrates, and prevalence of AtzD from *Pseudomonas* sp strain ADP. *Appl Environ Microb* 69:3653-3657
744

745 Fuentes S, Barra B, Caporaso JG, Seeger M (2016) From Rare to Dominant: A Fine-Tuned Soil Bacterial
746 Bloom during Petroleum Hydrocarbon Bioremediation. *Appl Environ Microb* 82:888-896
747

748 Giardina MC, Giardi MT, Filacchioni G (1982) Atrazine metabolism by *Nocardia*: elucidation of initial pathway
749 and synthesis of potential metabolites. *Agr Biol Chem* 46:1439-1445
750

751 Heap IM (2016) International Survey of Herbicide-Resistant Weeds. <http://www.weedscience.com>. Accessed:
752 06 March 2016
753

754 Houot S, Topp E, Yassir A, Soulas G (2000) Dependence of accelerated degradation of atrazine on soil pH in
755 French and Canadian soils. *Soil Biol & Biochem* 32:615-625
756

757 Howell CC, Semple KT, Bending GD (2014) Isolation and characterisation of azoxystrobin degrading bacteria
758 from soil. *Chemosphere* 95:370-378
759

760 Itoh H, Navarro R, Takeshita K., Tago K, Hayatsu M, Hori T, Kikuchi Y (2014) Bacterial population succession
761 and adaptation affected by insecticide application and soil spraying history. *Front Microb* 5:1-12
762

763 Katayama A, Bhula R, Burns GR, Carazo E, Felsot A, Hamilton D, Harris C, Kim YH, Kleter G, Koedel W,
764 Linders J, Peijnenburg JG, Sabljic A, Stephenson RG, Racke DK, Rubin B, Tanaka K, Unsworth J, Wauchope
765 RD (2010) Bioavailability of Xenobiotics in the Soil Environment. *Rev Environ Contam T* 203:1-86
766

767 Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glockner FO (2013) Evaluation of general
768 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies.
769 *Nucleic Acids Res* 41:e1
770

771 Krutz LJ, Burke IC, Reddy KN, Zablutowicz RM (2008) Evidence for cross-adaptation between s-triazine
772 herbicides resulting in reduced efficacy under field conditions. *Pest Manag Sci* 64:1024-1030.
773

774 Krutz LJ, Shaner DL, Zablotowicz RM (2010) Enhanced degradation and soil depth effects on the fate of
775 atrazine and major metabolites in Colorado and Mississippi soils. *J Environ Qual* 39:1369-77
776
777 LatLong. (2012-2014) <http://www.latlong.net/>. Accessed 10 March 2014
778
779 Mandelbaum RT, Allan DL, Wackett LP (1995) Isolation and Characterisation of a *Pseudomonas* Sp. that
780 Mineralizes the s-triazine Herbicide Atrazine. *Appl Environ Microb* 61:1451-1457
781
782 Mueller TC, Steckel LE, Radosevich M (2010) Effect of Soil pH and Previous Atrazine Use History on Atrazine
783 Degradation in a Tennessee Field Soil. *Weed Sci* 58:478-483
784
785 Mulbry WW, Zhu H, Nour SM, Topp E (2002) The triazine hydrolase gene *trzN* from *Nocardioides* sp strain
786 C190: Cloning and construction of gene-specific primers. *Fems Microbiol Lett* 206:75-79
787
788 Ngigi AN, Getenga ZM, Dorfler U, Boga HI, Kuria B, Ndalu P, Schroll R (2013) Effects of carbon amendment
789 on in situ atrazine degradation and total microbial biomass. *J Environ Sci Health B* 48:40-8
790
791 Nicol GW, Leininger S, Schleper C, Prosser JI (2008) The influence of soil pH on the diversity, abundance and
792 transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ Microbiol* 10:2966-2978
793
794 OECD. 2000. Test No. 106: Adsorption -- Desorption Using a Batch Equilibrium Method. OECD Publishing
795
796 OECD. 2002. Test No. 307: Aerobic and Anaerobic Transformation in Soil. OECD Publishing
797
798 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MH,
799 Wagner H (2013) *vegan: Community Ecology Package*. Accessed 24 July 2014
800
801 Orlikowska A, Fisch K, Schultz-Bull, DE (2015) Organic polar pollutants in surface waters of inland seas. *Mar*
802 *Pollut Bull* 101:860-866

803 Piutti S, Marchand AL, Lagacherie B, Martin-Laurent F, Soulas G (2002) Effect of cropping cycles and
804 repeated herbicide applications on the degradation of diclofopmethyl, bentazone, diuron, isoproturon and
805 pendimethalin in soil. *Pest Manag Sci* 58:303-312
806

807 Popov VH, Cornish PS, Sultana K, Morris EC (2005) Atrazine degradation in soils: the role of microbial
808 communities, atrazine application history, and soil carbon. *Aust J Soil Res* 43:861-871
809

810 Racke KD (1990) Pesticides in the Soil Microbial Ecosystem. *ACS Symposium Series* 426:1-12
811 Sagarkar S, Mukherjee S, Nousiainen A, Bjorklof K, Purohit HJ, Jorgensen KS, Kapley A (2013) Monitoring
812 bioremediation of atrazine in soil microcosms using molecular tools. *Environ Pollut* 172:108-115
813

814 Schroll R, Becher HH, Dorfler U, Gayler S, Hartmann HP, Ruoss J (2006) Quantifying the effect of soil
815 moisture on the aerobic microbial mineralization of selected pesticides in different soils. *Envir Sci & Tech*
816 40:3305-3312
817

818 Seffernick JL, Aleem A, Osborne JP, Johnson G, Sadowsky MJ, Wackett LP (2007) Hydroxyatrazine N-
819 ethylaminohydrolase (AtzB): an amidohydrolase superfamily enzyme catalyzing deamination and
820 dechlorination. *J Bacteriol* 189:6989-6997
821

822 Shaner DL, Henry WB, Krutz LJ, Hanson B (2007) Rapid assay for detecting enhanced atrazine degradation in
823 soil. *Weed Sci* 55:528-535
824

825 Shao ZQ, R Behki (1996) Characterization of the expression of the *thcB* gene, coding for a pesticide-degrading
826 cytochrome P-450 in *Rhodococcus* strains. *Appl Environ Microb* 62:403-407
827

828 Shapir N, Mongodin EF, Sadowsky MJ, Daugherty SC, Nelson KE, Wackett LP (2007) Evolution of catabolic
829 pathways: Genomic insights into microbial s-triazine metabolism. *J Bacteriol* 189:674-682
830

831 Shapir N, Rosendahl C, Johnson G, Andreina M, Sadowsky MJ, Wackett LP (2005) Substrate specificity and
832 colorimetric assay for recombinant TrzN derived from *Arthrobacter aurescens* TC1. *Appl Environ Microb*
833 71:2214-2220
834

835 Singh BK, Walker A, Morgan JA, Wright DJ (2003) Effects of soil pH on the biodegradation of chlorpyrifos
836 and isolation of a chlorpyrifos-degrading bacterium. *Appl Environ Microb* 69:5198-5206
837 Sniegowski K, Bers K, Ryckeboer J, Jaeken P, Spanoghe P, Springael D (2012) Minimal pesticide primed soil
838 inoculum density to secure maximum pesticide degradation efficiency in on-farm biopurification systems.
839 *Chemosphere*. 88:1114-1118
840

841 Strong LC, Rosendahl C, Johnson G, Sadowsky MJ, Wackett LP (2002) *Arthrobacter aurescens* TC1
842 metabolizes diverse s-triazine ring compounds. *Appl Environ Microb* 68:5973-5980
843

844 SunEarthTools.com (2009-2016) <http://www.sunearthtools.com/>. Accessed 21 October 2016
845

846 Syngenta (2016) Atrazine and the EU. http://www.atrazine.com/sciencesafety/atrazine_safety_eu.aspx.
847 Accessed 23 February 2016.
848

849 Tomlin CDS (2009) *The pesticide manual: A World compendium*. Alton: BCPC
850

851 Topp E, Mulbry WM, Zhu H, Nour SM, Cuppels D (2000) Characterization of S-triazine herbicide metabolism
852 by a *Nocardioide* sp isolated from agricultural soils. *Appl Environ Microb* 66:3134-3141
853

854 Udikovic-Kolic N, Martin-Laurent F, Devers M, Petric I, Kolar AB, Hrsak D (2008) Genetic potential, diversity
855 and activity of an atrazine-degrading community enriched from a herbicide factory effluent. *J Appl Microbiol*
856 105:1334-1343
857

858 Udikovic-Kolic N, Hrsak D, Devers M, Klepac-Ceraj V, Petric I, Martin-Laurent F (2010) Taxonomic and
859 functional diversity of atrazine-degrading bacterial communities enriched from agrochemical factory soil. *J*
860 *Appl Microbiol* 109:355-367.

861 Udikovic-Kolic N, Devers-Lamrani M, Petric I, Hrsak D, Martin-Laurent F (2011) Evidence for taxonomic and
862 functional drift of an atrazine-degrading culture in response to high atrazine input. *Appl Microbiol Biot*
863 90:1547-1554
864

865 Udikovic-Kolic N, Scott C, Martin-Laurent F (2012) Evolution of atrazine-degrading capabilities in the
866 environment. *Appl Microbiol Biot* 96:1175-1189
867

868 Walker ARSJ (1993) Degradation, Biodegradation and Enhanced Biodegradation. In *Proc 9th Symp Pesticide*
869 *Chemistry: The chemistry, mobility and degradation of xenobiotics*. Piacenza, Italy
870

871 Walkley A, Black IA (1934) An examination of the degtjareff method for determining soil organic matter, and a
872 proposed modification of the chromic acid titration method. *Soil Sci* 37:29-38
873

874 Wang J, Zhu L, Wang Q, Wang J, Xie H (2014) Isolation and Characterization of Atrazine Mineralising
875 *Bacillus subtilis* Strain HB-6. *PLOS one* 9, 9 e107270:1-9
876

877 Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE (2002) Quantitative Multiprobe PCR
878 Assay for Simultaneous Detection and Identification to Species Level of Bacterial Pathogens. *J Clin Microbiol*
879 40:3449-3454
880

881 Yanze-Kontchou C, Gschwind N (1994) Mineralization of the herbicide atrazine as a carbon source by a
882 *Pseudomonas* strain. *Appl Environ Microb* 60:4297-4302
883

884 Zablotowicz RM, Krutz LJ, Reddy KN, Weaver MA, Koger CH, Locke MA (2007) Rapid development of
885 enhanced atrazine degradation in a dundee silt loam soil under continuous corn and in rotation with cotton. *J*
886 *Agr Food Chem* 55:852-859

Figures

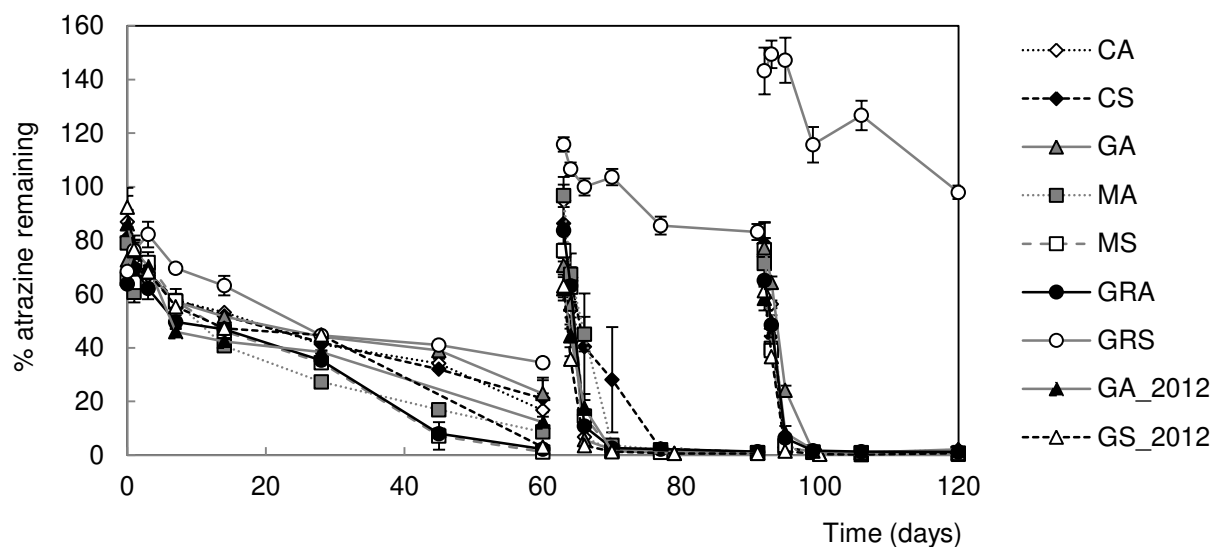


Fig. 1: Dissipation of atrazine over three applications to nine temperate soils. Atrazine concentration in soil subsamples was monitored at regular intervals by HPLC-UV. Error bars show the standard error between replicates, $n = 12$ for GA_2012 & GS_2012 applications 1 and 2, $n = 6$ for application 3, $n = 4$ for all other soils. Soil identifier; CA: Cotril agricultural CS: Cotril set aside, GA: Ganthorpe agricultural MA: Mount agricultural, MS: Mount set aside, GRA: Grange agricultural, GRS: Grange set aside, GA_2012: Ganthorpe agricultural collected in 2012 and GS_2012: Ganthorpe set aside collected in 2012

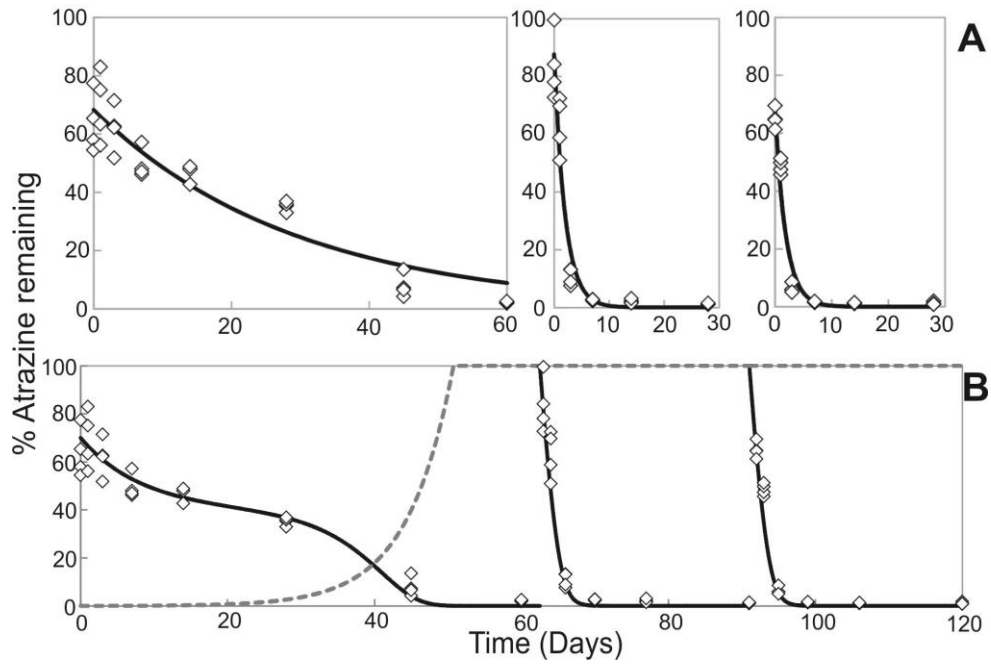


Fig. 2: Comparison of modelling approaches for the dissipation of atrazine in GRA over three applications. Using the regulatory single first order (SFO) approach (A), with each application modelled separately and the ‘growth-linked model’ described in this study (B). The growth-linked model enabled all applications to be modelled simultaneously. In both modelling approaches the model fit of % atrazine remaining is shown as a solid black line and individual soil sub-samples as diamonds ($n = 4$). For the growth-linked model (B) the grey dashed line represents the number of atrazine degraders

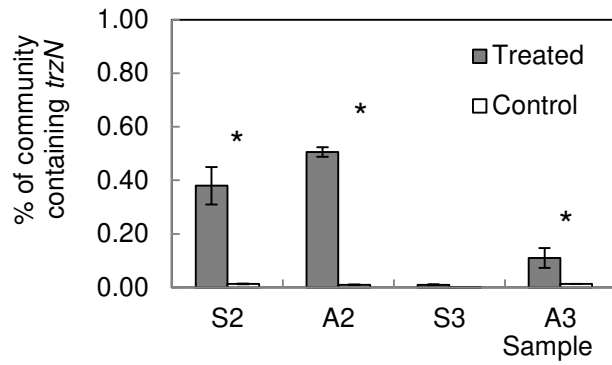


Fig. 3: Percentage of the bacterial community that contain the atrazine degrading gene *trzN* in the GA_2012 and GS_2012 soils. The *trzN* gene was monitored in the Ganthorpe agricultural soil; GA_2012 (A) and Ganthorpe set aside soil; GS_2012 (S) soils fourteen days after the second (2) or third application (3) of atrazine to each soil. *TrzN* was measured in atrazine treated and control sub-samples. The proportions of the community carrying *trzN* was normalised against the *16S rRNA* gene for each sample. Error bars show the standard error between experimental replicates, n = 6. The significant differences between the proportion of the community containing *trzN* between treated and control soils are indicated by * ($p < 0.05$)

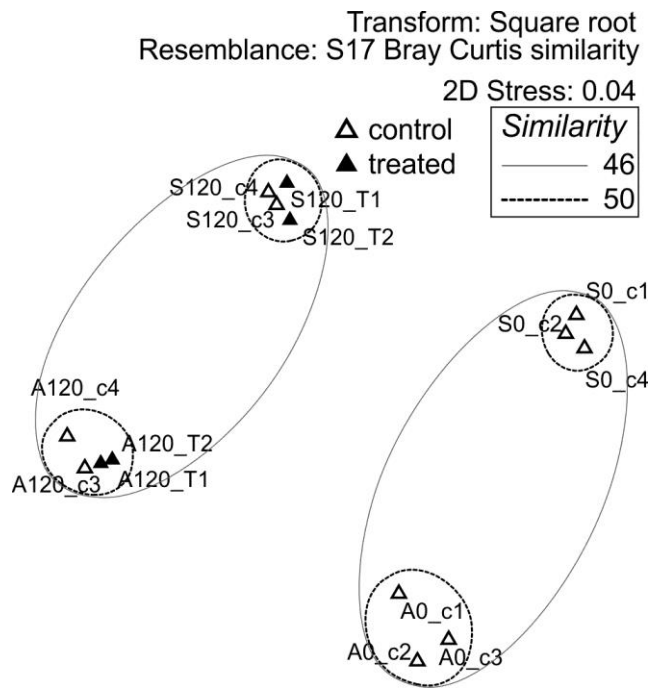


Fig. 4: Non-metric multidimensional scaling plot of the association of bacterial communities with atrazine treatment in the GA_2012 and GS_2012 soils. Each bacterial community is represented by a triangle, originating from the Ganthorpe agricultural soil; GA_2012 (A) and Ganthorpe set aside soil; GS_2012 (S). The bacterial communities are based on OTU clustering of the pyrosequencing of *16S rRNA* genes. The variables included in the analysis were soil history: set aside (S) or agricultural (A), duration in days under incubation conditions (0 or 120 days) and atrazine treatment: treated (T) or control (C). The similarity ellipses are based on hierarchical clustering shown in the Online Resource 26

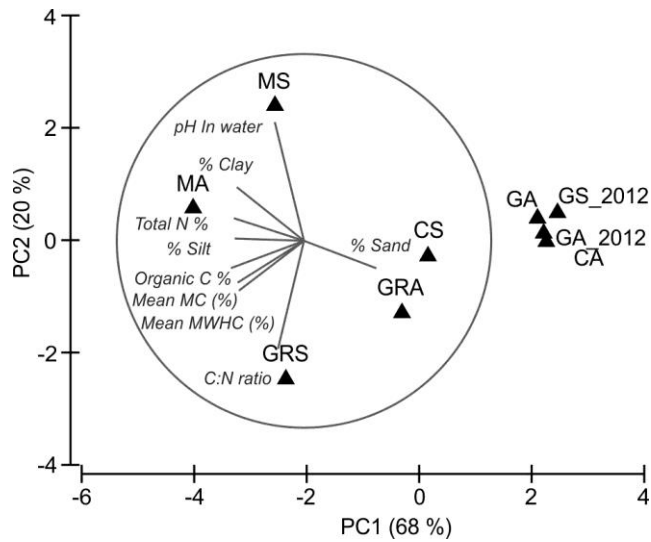


Fig. 5: Principal Component Analysis (PCA) of the association of nine temperate soils with various physio-chemical properties. Measured soil properties were normalised and the corresponding data matrix was subject to PCA. Each triangle represents an individual soil. The association between different soils is plotted along the first two principal components, which represent 68 % and 20 % of the variation between the soils. Soil properties; MWHC; maximum water holding capacity, MC; moisture content, C:N ratio; carbon : nitrogen ratio and total N; total nitrogen. Soil identifier; CA: Cotril agricultural CS: Cotril set aside, GA: Ganthorpe agricultural MA: Mount agricultural, MS: Mount set aside, GRA: Grange agricultural, GRS: Grange set aside, GA_2012: Ganthorpe agricultural collected in 2012 and GS_2012: Ganthorpe set aside collected in 2012

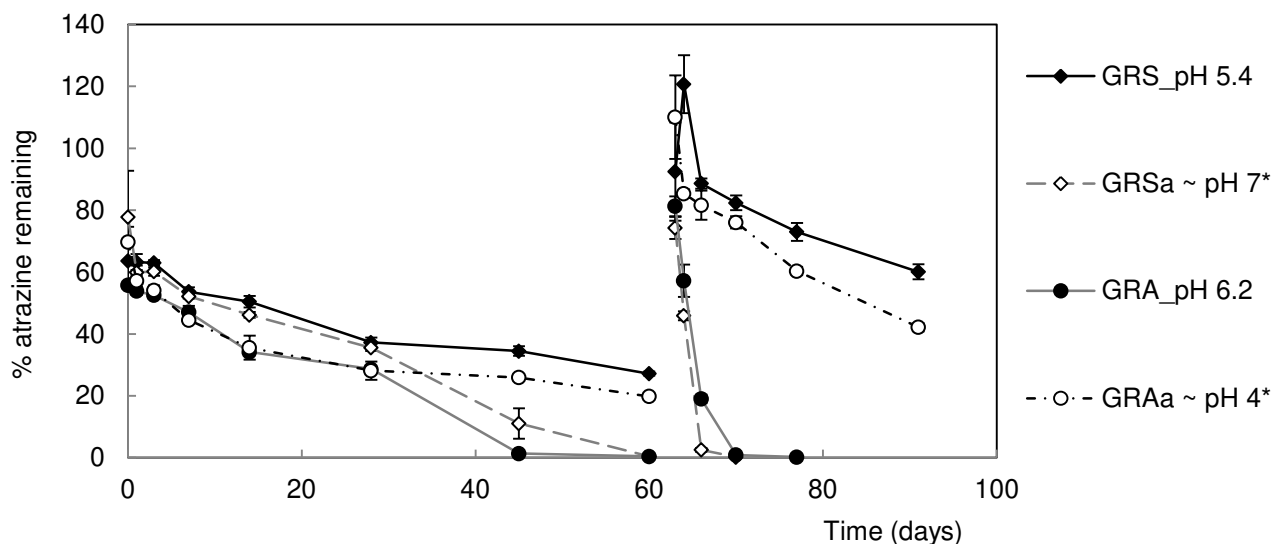


Fig. 6: Effect of soil pH on atrazine dissipation over two applications in the GRA_pH and GRS_pH soils. Atrazine was applied to the Grange set aside soil (GRS_pH) and Grange agricultural soil (GRA_pH) which were collected in 2014 and their pH was amended (-a) to approximately pH 7 (GRSa) and pH 4 (GRAa), respectively. Error bars represent the standard error between replicates (n = 4). Parameters used for the SFO model fits are provided in the Online Resource 10, * shows that the pH of the amended soils (GRSa and GRAa) are only approximate as there was minor variation in their soil pH throughout the experiment (Online Resource 4)

Tables

Table 1. Identification and properties of the soils used in this study.

| <i>Soil#</i> | <i>Farm</i> | <i>Year collected</i> | <i>Soil history[§]</i> | <i>Textural class (USDA)</i> | <i>Sand (%)</i> | <i>Silt (%)</i> | <i>Clay (%)</i> | <i>Total N (%)</i> | <i>OC (%)</i> | <i>C:N ratio</i> | <i>pH (In H₂O)</i> | <i>Moisture content (g water)[^]</i> |
|--------------|-------------|-----------------------|---------------------------------|------------------------------|-----------------|-----------------|-----------------|--------------------|---------------|------------------|-------------------------------|---|
| GA_2012* | Ganthorpe | 2012 | A | SL | 79 | 12 | 9 | 0.14 | 1.4 | 10 | 6.39 | 0.12 |
| GS_2012* | | | S | SL | 81 | 8 | 11 | 0.14 | 1.4 | 10 | 6.26 | 0.07 |
| GA | Ganthorpe | | A | SL | 79 | 10 | 11 | 0.11 | 1.2 | 10.9 | 6.55 | 0.15 |
| CS | Cotril | | S | SL | 77 | 12 | 11 | 0.19 | 1.9 | 10 | 6.32 | 0.22 |
| CA | Cotril | | A | LS | 83 | 8 | 9 | 0.12 | 1.3 | 10.8 | 6.64 | 0.16 |
| MS | Mount | 2013 | S | SCL | 54 | 24 | 22 | 0.23 | 2.2 | 9.6 | 8.14 | 0.22 |
| MA | Mount | | A | SCL | 51 | 24 | 25 | 0.24 | 2.8 | 11.7 | 7.43 | 0.25 |
| GRS | Grange | | S | SL | 65 | 20 | 15 | 0.21 | 2.7 | 12.9 | 5.43 | 0.29 |
| GRA | Grange | | A | SL | 67 | 22 | 11 | 0.14 | 1.9 | 13.6 | 6.53 | 0.20 |
| GRA_pH** | Grange | 2014 | A | SL | 62 | 24 | 14 | 0.16 | 1.4 | 8.8 | 6.23 | 0.18 |
| GRS_pH** | | | S | SCL | 55 | 26 | 19 | 0.33 | 3.7 | 11.2 | 5.39 | 0.23 |

Atrazine degradation and the presence of the atrazine degrading genes was monitored in all soils

*soils used to monitor microbial community changes (ATP, Q-PCR and Pyrosequencing)

**soils used to monitor the effect of pH on accelerated degradation

[§]S; set aside soil, A; agricultural soil

[^] per g of dry soil (105 °C)

Table 2. Primers used for amplification of the atrazine degrading genes in PCR and Q-PCR. The hydrolytic enzymes that are encoded by each gene are; AtzA/TrzN: atrazine chlorohydrolase; AtzB: hydroxyatrazine hydrolase; AtzC: N-isopropylammelide hydrolase; AtzD/TrzD: cyanuric acid hydrolase; AtzE: biuret hydrolase and AtzF: Allophanate hydrolase.

| <i>Gene</i> | <i>Amplicon Length (bp)</i> | <i>Primer name</i> | <i>Primer sequence</i> | <i>Annealing Temp. (°c)</i> | <i>Reference</i> |
|-------------|-----------------------------|--------------------|------------------------|-----------------------------|----------------------|
| <i>atzA</i> | 500 | atzA_F | CCATGTGAACCAGATCCT | 55.7 | De Souza et al. 1998 |
| | | atzA_R | TGAAGCGTCCACATTACC | | |
| <i>trzN</i> | 400 | Trz_Nf, C190-10 | CACCAGCACCTGTACGAAGG | 59 | Mulbry et al. 2002 |
| | | Trz_Nr, C190-11 | GATTCGAACCATTCCAAACG | | |
| <i>atzB</i> | 500 | atzB_F | TCACCGGGGATGTCGCGGGC | 62.4 | De Souza et al. 1998 |
| | | atzB_R | CTCTCCCGCATGGCATCGGG | | |
| <i>atzC</i> | 600 | atzC_F | GCTCACATGCAGGTACTCCA | 62.4 | De Souza et al. 1998 |
| | | atzC_R | GTACCATATCACCGTTTGCCA | | |
| <i>atzD</i> | 202 | atzD_F | TCCCACCTGACATCACAAAC | 62.4 | Devers et al. 2004 |
| | | atzD_R | GGGTCTCGAGGTTTGATTG | | |
| <i>trzD</i> | 663 | TrzD_F | CACTGCACCATCTTCACC | 55 | Fruchey et al. 2003 |
| | | TrzD_R | GTTACGAAC CTCACCGTC | | |
| <i>atzE</i> | 203 | atzE_F | GAGCCTCTGTCCGTAGATCG | 60 | Devers et al. 2004 |

| | | | | | |
|------------------|-----|------------|------------------------|----|--------------------|
| | | atzE_R | GATGGCGTGTACCGTTTACC | | |
| <i>atzF</i> | 233 | atzF_F | ACCAGCCCTTGAATCATCAG | 57 | Devers et al. 2004 |
| | | atzF_R | TATTGTCCCGATACCCAACG | | |
| <i>16_Q rRNA</i> | 161 | 16S_qPCR_F | TGGAGCATGTGGTTTAATTCGA | - | Yang et al. 2002 |
| | | 16S_qPCR_R | TGCGGGACTTAACCCAACA | | |
| <i>TrzN_Q</i> | 70 | TrzN_Q_F | GCTTCTGCGACGACCTGTTC | - | In this study |
| | | TrzN_Q_R | TGGTCGATGAGACCCAG | | |

Table 3. Estimated time for 50% of atrazine to degrade in days (DT₅₀) and corresponding Chi squared value (χ^2) for single first order (SFO) fits for atrazine dissipation in soils that received two or three successive applications of atrazine. Refer to Table 1 for soil origin.

| <i>Soil</i> | <i>First application</i> | | <i>Second application</i> | | <i>Third application</i> | |
|-------------|--------------------------|----------------------|---------------------------|----------------------|--------------------------|----------------------|
| | <i>DT₅₀</i> | <i>X₂</i> | <i>DT₅₀</i> | <i>X₂</i> | <i>DT₅₀</i> | <i>X₂</i> |
| GS_2012 | 21.49 | 10.26 | 1.76 | 9.16 | 1.53 | 22.24 |
| GA_2012 | 20.18 | 11.85 | 1.08 | 6.73 | 1.01 | 16.37 |
| CS | 32.34 | 5.72 | 2.93 | 12.42 | 0.98 | 12.8 |
| CA | 30.23 | 8.2 | 1.03 | 10.08 | 1.3 | 26.9 |
| MS | 19.81 | 11.09 | 1.59 | 17.15 | 0.91 | 8.8 |
| MA | 19.58 | 7.84 | 2.72 | 5.57 | 0.97 | 12.13 |
| GRS | 48.14 | 6.58 | 57.88 | 5.66 | 48.62 | 5.06 |
| GRA | 20.38 | 10.39 | 1.39 | 16.73 | 1.32 | 18.61 |
| GA | 37.1 | 10.94 | 1.13 | 15.04 | 1.89 | 12.26 |
| GRS_pH* | 46.08 | 3.81 | 32.61 | 9.45 | - | - |
| GRSa** | 18.25 | 9.78 | 1.05 | 13.94 | - | - |
| GRA_pH* | 18.04 | 11.77 | 1.54 | 6.64 | - | - |
| GRAa** | 30.02 | 10.83 | 21.29 | 7.36 | - | - |

*soils collect from Grange farm in 2014 for the pH experiment

**GRSa and GRAa soils originated from GRA_pH and GRS_pH after their pH was amended

Table 4. Summary of the atrazine degrading genes detected in nine temperate soils, 14 days after the third application of atrazine. Refer to Table 1 for soil origin. Refer to Table 2 for a description of the hydrolytic enzymes that are encoded by each gene.

| Soil | Number of genes detected | <i>Number</i> | | | | | | | |
|-------------|--------------------------------|------------------------|------------------------|------------------------|------------------------|-------------|------------------------|-------------|-------------|
| | | <i>atzA</i> (100 %) | <i>trzN</i> (100 %) | <i>atzB</i> (100 %) | <i>atzC</i> (100 %) | <i>atzD</i> | <i>trzD</i> (100 %) | <i>atzE</i> | <i>atzF</i> |
| GA_201 2 | 3 | | + | + | | | + | | |
| GS_201 2 | 4 | + | + | + | | | + | | |
| CS | 3 | + | + | + | | | | | |
| CA | 3 | + | + | + | | | | | |
| MS | 1 | | + | | | | | | |
| MA | 3 | + | + | + | | | | | |
| GRS | 0 | | | | | | | | |
| GRA | 3 | + | + | + | | | | | |
| GA | 4 | + | + | + | + | | | | |

*The closest relative for each of the atrazine degrading genes detected are shown in online resource 20