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

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

**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-etylamino-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 (Zablotowicz 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 $H_2O$ 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 °C ± 2 °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 °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 ($Ca(OH)_2$) added to maintain ~ 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 ($Al_2(SO_4)_3$) added
145 and was maintained at ~ 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 µg $g^{-1}$ 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 µg $mL^{-1}$. 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 µg $mL^{-1}$ to 5.0 µg $mL^{-1}$. 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, $\chi^2$ 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 ($\chi^2$)
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;

193

$$[Atr](t+n) = [Atr]t - \left(Y \times n \times k_1 \times e^{-k1t}\right) - \left(N \times n \times V \times [Atr] \div \left([Atr] + K_s\right)\right)$$

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:

202 $$N = N_0^{e\mu t}$$

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 $CaCl_2$ 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 $CaCl_2$ was added to ensure all tubes had

219 25 mL of 0.01 M $CaCl_2$, 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 $$C_S = K_f \times C_e^{1/n}$$

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 GA_2012 and GS_2012 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

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

### 2.10. DNA extraction & PCR

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

### 2.11. Detection & sequencing of atrazine degrading genes

Samples from different time points across the three applications of atrazine (2 applications for the pH study) were checked for atrazine degrading genes (Online Resource 1). The PCR mix consisted of 1x KAPA HiFi fidelity buffer (Kapa Biosystems, Woburn, MA, USA), 0.3 µM of dNTPs, 0.3 µM of each primer (Table 2), 1 U $\mu L^{-1}$ KAPA HiFi polymerase and nuclease-free water (Severn biotech Ltd, Worcestershire, UK) to reach 25 µL final volume. The PCR followed these thermal cycling conditions: initial denaturation at 95 °C for 5 min, and 30 cycles of denaturation at 98 °C for 30 sec, annealing for 15 sec (at the specified temperature in Table 2) and 15 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 µg $mL^{-1}$ 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 ng $\mu l^{-1}$ 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 $$\frac{\text{trzN gene concentration}}{\text{16S rRNA gene concentration}} \times \frac{\text{length of 16S gene product (bp)}}{\text{length of trzN gene product (bp)}} \times 100$$

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 **<u>CACGACGTTGTAAAACGAC</u>**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 **CCATCTCATCCCTGCGTGTCTCCGAC**TCAGNNNN**CACGACGTTGTAAAACGAC** -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 $\mu L^{-1}$ 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 (Oksannen 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 $\mu g.L^{-1}$), i.e $K_s = 20.9$ μM. This result from modelling is remarkable
436 given the atrazine degrading enzyme TrzN has been measured to have $K_m = 20$ μM (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

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

### 3.4 GRS was most strongly associated with pH

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

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

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

PC2 is mainly explained by pH, although there is some contribution from carbon to nitrogen ratio (C:N) and moisture content too, while PC3 is mainly explained by C:N ratio (Online Resource 23). Based on the principal component scores, it was clear that the variation between the agricultural soils; CA, GA and MA were mainly dependent on PC1 (Online Resource 24). Specifically, CA had the highest sand content, while GA and MA had the lowest and highest organic carbon contents, respectively (Table 1). GRA was an outlier from the other soils mainly affiliated with PC3 (Online Resource 24) and explained by having the highest C:N ratio (Table 1), whereas MS and GRS were strongly affiliated with PC2, due to them having the highest and lowest pH 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 $DT_{50}$ 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 $DT_{50}$ 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 (Krutz 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

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**Figures**

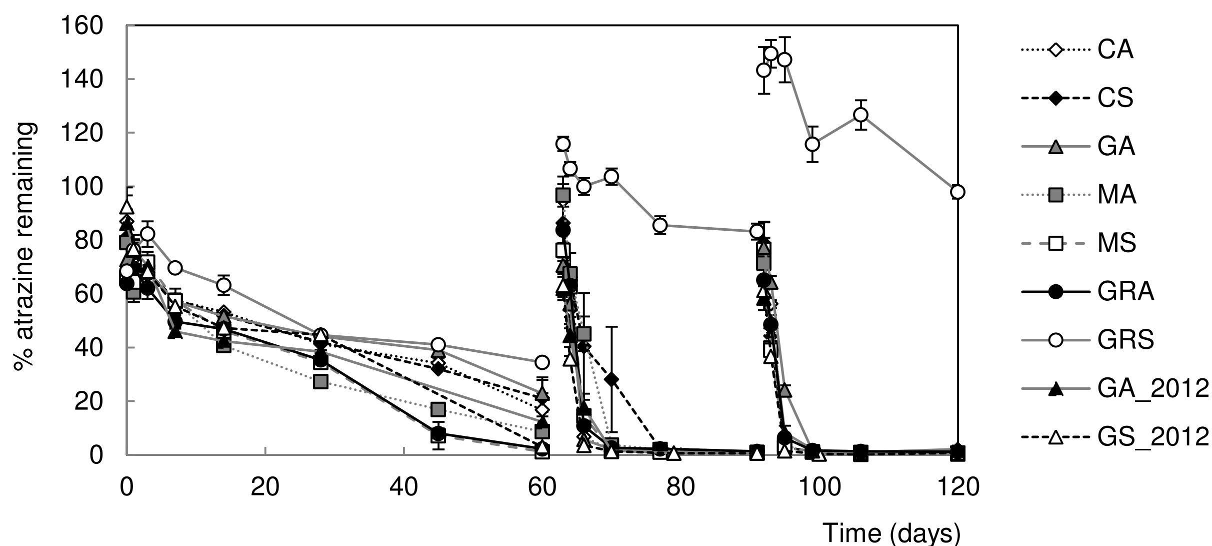


**Fig. 1:** Dissipation of atrazine over three applications to nine temperate soils. Atrazine concentration in soil subsamples was monitored at regular intervals by HPLC-UV. Error bars show the standard error between replicates, n = 12 for GA_2012 & GS_2012 applications 1 and 2, n = 6 for application 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

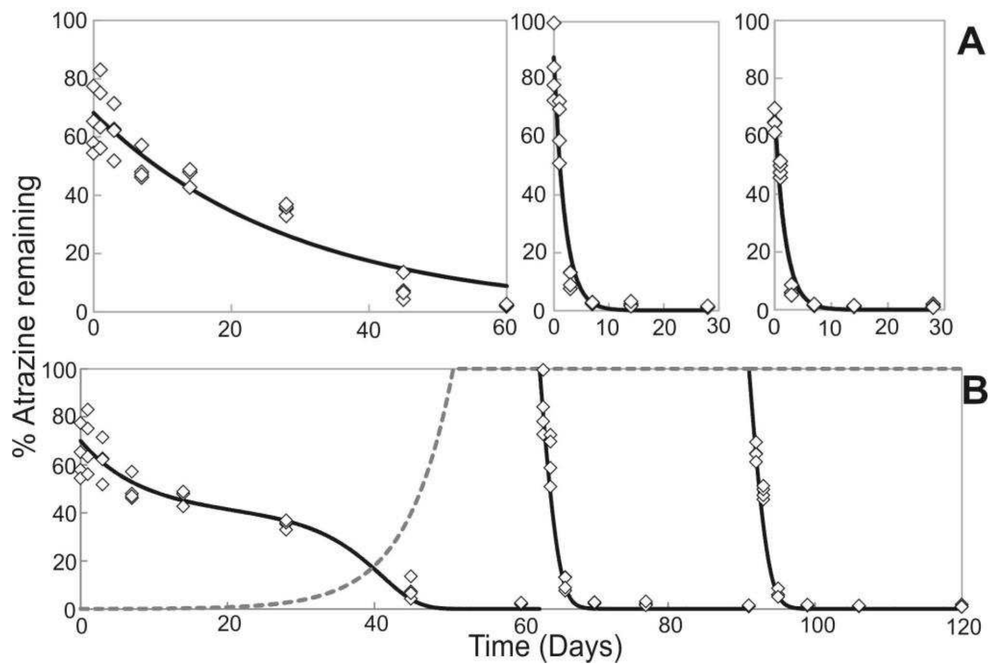


**Fig. 2:** Comparison of modelling approaches for the dissipation of atrazine in GRA over three applications. Using the regulatory single first order (SFO) approach (A), with each application modelled separately and the 'growth-linked model' described in this study (B). The growth-linked model enabled 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

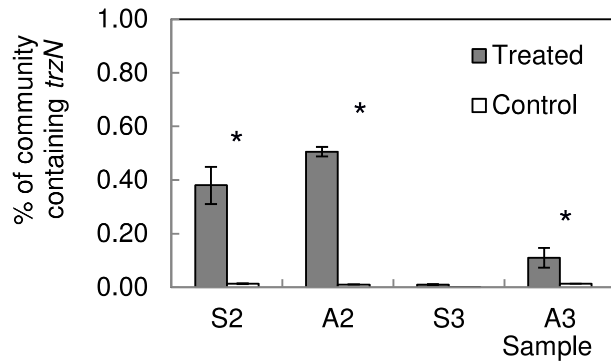


**Fig. 3:** Percentage of the bacterial community that contain the atrazine degrading gene trzN in the GA_2012 and GS_2012 soils. The trzN gene was monitored in the Ganthorpe agricultural soil; GA_2012 (A) and Ganthorpe set aside soil; GS_2012 (S) soils fourteen days after the second (2) or third application (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 * ($p < 0.05$)

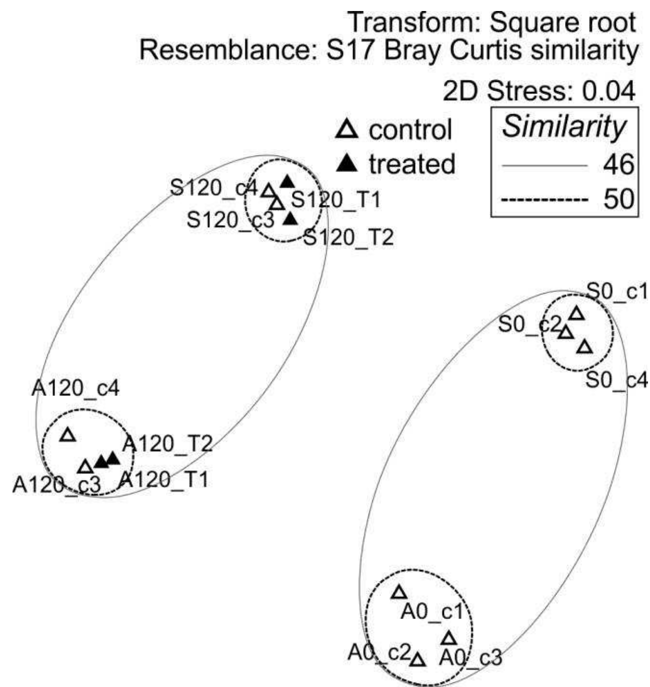


**Fig. 4:** Non-metric multidimensional scaling plot of the association of bacterial communities with atrazine treatment in the GA_2012 and GS_2012 soils. Each bacterial community is represented by a triangle, originating from the Ganthorpe agricultural soil; GA_2012 (A) and Ganthorpe set aside soil; 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

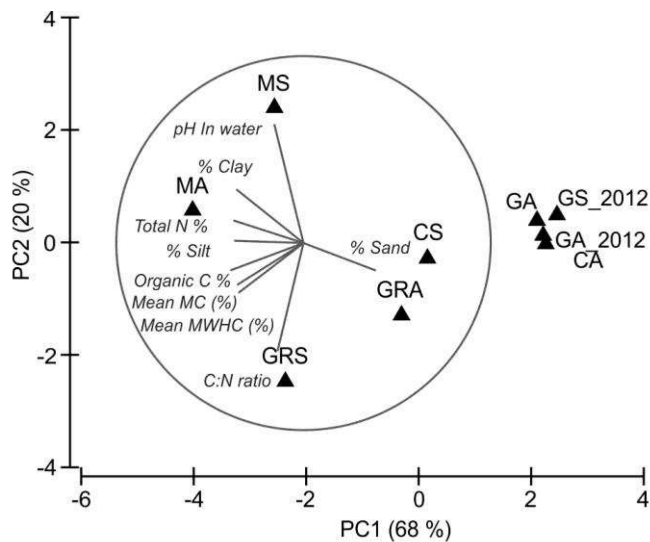


**Fig. 5:** Principal Component Analysis (PCA) of the association of nine temperate soils with various physio-chemical properties. Measured soil properties were normalised and the corresponding data matrix was subject to PCA. Each triangle represents an individual soil. The association between different 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

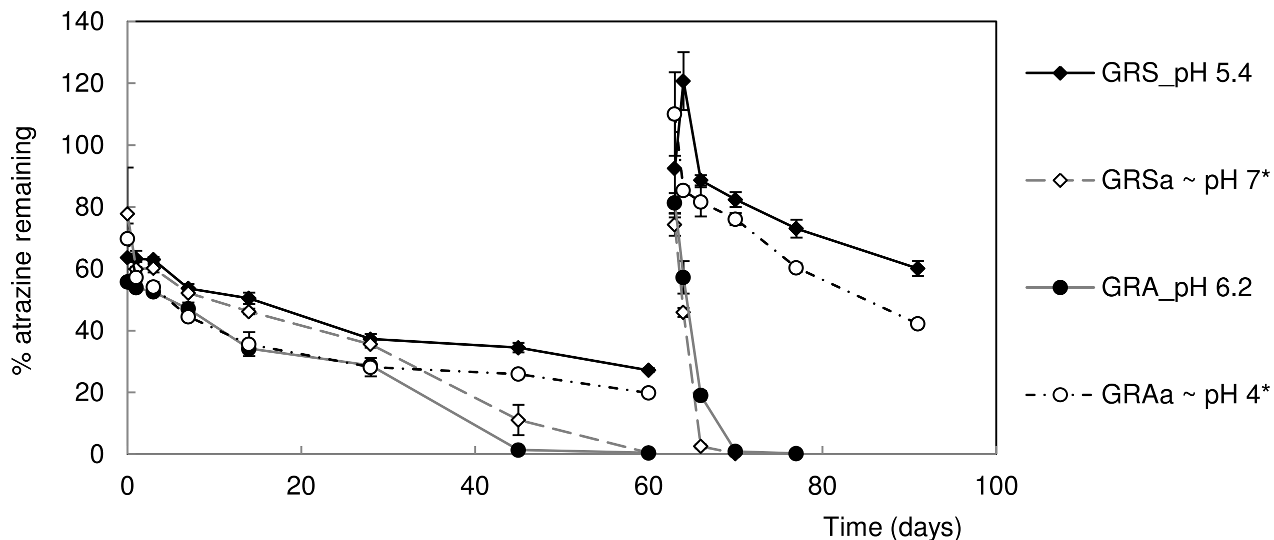


**Fig. 6:** Effect of soil pH on atrazine dissipation over two applications in the GRA_pH and GRS_pH soils. Atrazine was applied to the Grange set aside soil (GRS_pH) and Grange agricultural soil (GRA_pH) which were collected in 2014 and their pH was amended (-a) to approximately pH 7 (GRSa) and pH 4 (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.**

| Soil# | Farm | Year collected | Soil history[$] | Textural class (USDA) | Sand (%) | Silt (%) | Clay (%) | Total N (%) | OC (%) | C:N ratio | pH (In $H_2O$) | Moisture content (g water)^ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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 | 2013 | 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 | | 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** | Grange | | 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.

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

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

**Table 3. Estimated time for 50% of atrazine to degrade in days ($DT_{50}$) and corresponding Chi squared value ($\chi^2$) 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.

| | **First application** | | **Second application** | | **Third application** | |
|---|---|---|---|---|---|---|
| **Soil** | **$DT_{50}$** | **$X_2$** | **$DT_{50}$** | **$X_2$** | **$DT_{50}$** | **$X_2$** |
| 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 * | atzA (100 %) | trzN (100 %) | atzB (100 %) | atzC (100 %) | atzD | trzD (100 %) | atzE | atzF |
|---|---|---|---|---|---|---|---|---|---|
| GA_2012 | 3 | | + | + | | | + | | |
| GS_2012 | 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