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1 # Labour sharing promotes coexistence in atrazine degrading bacterial
2 communities

3
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13 ***Keywords:*** Black Queen Hypothesis, atrazine biodegradation, public goods, coexistence

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15 **SUMMARY**

16 Microbial communities exert a pivotal role in the biodegradation of xenobiotics including
17 pesticides[1]. In the case of atrazine, multiple studies have shown that its degradation involved a
18 consortia rather than a single species[2,3,4,5], but little is known about how interdependency between
19 the species composing the consortium is set up. The Black Queen Hypothesis (BQH) formalized
20 theoretically the conditions leading to the evolution of dependency between species[6]: members of
21 the community called 'helpers' provide publicly common goods obtained from the costly
22 degradation of a compound, while others called 'beneficiaries' take advantage of the public goods,
23 but lose access to the primary resource through adaptive degrading gene loss. Here, we test whether
24 liquid media supplemented with the herbicide atrazine could support coexistence of bacterial
25 species through BQH mechanisms. We observed the establishment of dependencies between species
26 through atrazine degrading gene loss. Labour sharing between members of the consortium led to
27 coexistence of multiple species on a single resource and improved atrazine degradation potential.
28 Until now, pesticide degradation has not been approached from an evolutionary perspective under
29 the BQH framework. We provide here an evolutionary explanation that might invite researchers to
30 consider microbial consortia, rather than single isolated species, as an optimal strategy for isolation
31 of xenobiotics degraders. Also, we anticipate that future research should focus on the
32 bioaugmentation with stabilized and tightly structured microbial degrading consortia as an effective
33 solution for *in situ* bioremediation of sites polluted with recalcitrant compounds.

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35 **TEXT**

36 Microorganisms in nature usually co-exist as communities whose complexity is under the influence
37 of local environmental conditions and interindividuals interactions. Spatially structured
38 environments, such as biofilms, are more prone to support coexistence of multiple species
39 consuming the same resource because access to the resource and its metabolic by-products is
40 conditioned by the structure of the environment[7,8]. Interactions of various nature between bacterial
41 species that stabilize the community diversity can then arise and persist, but are most likely to
42 disappear when the structure is disrupted[9,10]. In mixed liquid cultures, competition for a single
43 resource is supposed to lead to competitive exclusion[11]. However, if the breaking down of the
44 resource includes both private and public goods, natural selection could favor the emergence of
45 dependency between species, and therefore coexistence, through adaptive gene loss according to the
46 Black Queen Hypothesis (BQH)[6,12].

47 Morris *et al.* formulated three key characteristics for a function to follow the BQH. The function
48 allowing consumption of the resource must be *i)* costly, *ii)* essential and *iii)* leaky. Atrazine is one of
49 the most heavily applied herbicide worldwide which is relatively persistent and mobile in soil, and
50 whose degradation products can be traced in soils decades after application[13,14]. Atrazine can be
51 mineralized by microorganisms and is a source of nitrogen. Its entire biodegradation pathway is
52 known (Fig. 1) and can be split into two parts: the upper part consisting in the dechlorination of
53 atrazine and the removal of the two lateral chains from the *s*-triazinic cycle, followed by the lower
54 part consisting in the complete degradation of cyanuric acid. Genes (*atz* and *trz* families) involved
55 in its mineralization are most often located on plasmids or on catabolic cassettes delimited by
56 insertion sequences[15]. Because of the cost of maintaining plasmids, atrazine genes are easily lost
57 after only a couple of generations in culture media without atrazine[16]. However, in nitrogen limited
58 environments, atrazine degrading capacities must be conserved because mineralization of atrazine
59 leads to the essential delivery of nitrogen[17]. Also, exchange of metabolites, such as

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60 desisopropylamine, aminoethanol and desethylamine, are produced and released in the environment
61 during atrazine degradation[18]. Therefore, atrazine biodegradation is a good candidate function to test
62 the BQH predictions, and in particular the emergence of dependency between species in a spatially
63 unstructured environment.

64 *Chelatobacter* sp. SR38[19], *Pseudomonas* sp. ADPe[16], *Arthrobacter* sp. TES[20] and *Variovorax* sp.
65 38R[21] are all originally soil bacteria isolated from arable soils exposed to atrazine. They are all able
66 to at least partly degrade either atrazine or its intermediary products (Fig. 1). They have all been
67 modified to resist to a different combination of two antibiotics in order to evaluate their frequency
68 in mixed culture on double-antibiotics plates. Based on their genetic repertoire for atrazine
69 degradation, it is quite straightforward to predict that in a liquid culture medium with atrazine as the
70 unique nitrogen source, *Arthrobacter* sp. TES and *Pseudomonas* sp. ADPe must coexist because
71 *Arthrobacter* sp. TES will degrade atrazine to supply its nitrogen needs and produce cyanuric acid
72 that will be used by *Pseudomonas* sp. ADPe as its nitrogen reservoir. However, predicting what
73 would happen in a more complex scenario where multiple competing strains evolve in
74 environments containing various nitrogen sources is not trivial. Here, we experimentally questioned
75 whether spatially unstructured atrazine-containing media could support coexistence of multiple
76 atrazine degrading species through BQH mechanisms.

77 We propagated for ~ 100 generations four-species consortia, in triplicate, in seven minimal media
78 supplemented with citrate in excess, as the carbon source, and either one among three nitrogen
79 sources: atrazine, cyanuric acid: the metabolic product of the upper part of the atrazine degradation
80 pathway, or ammonium sulfate; or 2-way and 3-way combinations of the above mentioned nitrogen
81 sources, keeping the N molarity constant. We found that out of the seven media, all species
82 coexisted in the four ones containing atrazine, *Arthrobacter sp. TES* going extinct in the three
83 atrazine-free media (Fig 2A). The extinction of *Arthrobacter sp. TES* in cyanuric acid supplemented
84 media was expected as it does not possess the metabolic pathway to degrade cyanuric acid, however

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85 its extinction also in ammonium sulfate supplemented media is more surprising. Intriguingly, the
86 equilibrium frequencies of the three other species, besides *Arthrobacter* sp. TES, are quite similar
87 after ~100 generations in six out of the seven media, with *Pseudomonas* sp. ADP dominant at ~10^7
88 CFU.mL^{-1} and the two remaining species at ~10^6 CFU.mL^{-1}, the exception being the ammonium
89 sulfate – cyanuric acid supplemented medium where *Variovorax* sp. 38R appeared to be dominant at
90 ~10^7 CFU.mL^{-1}.

91 We then assessed using multiplex PCRs the presence of the atrazine degradation genes in the
92 evolved consortia across the seven different media. We found that in the four media containing
93 atrazine, all initially present *atz* or *trz* genes were kept in the evolved consortia, preluding the
94 conservation of the atrazine mineralisation potential in these environments (Fig 2B). In the atrazine-
95 free media, only *atzA*, responsible for the dechlorination of atrazine, a step that releases chlorine but
96 does not provide any nitrogen input to cells, and *atzD*, responsible for the first step of the
97 degradation of cyanuric acid, were conserved in the three environments while *atzC* and *trzD* were
98 specifically kept in the ammonium sulfate – cyanuric acid supplemented medium. Since in that
99 specific medium, *atzC* does not confer any advantage to its carrier, *Chelatobacter sp. SR38,* it has
100 likely been carried along because of its colocalization with *trzD* on the same plasmid[22], responsible
101 for the degradation of cyanuric acid. Rapid evolution, probably through the loss of *atz* genes
102 containing plasmids, therefore led to the loss of the atrazine degradation function in atrazine-free
103 environments, confirming the genetic load of maintaining atrazine genes in atrazine uncontaminated
104 environments.

105 We then evaluated the atrazine mineralization potential, using ^{14}C-atrazine either labelled on the
106 ethylamino chain or on the *s*-triazinic cycle, of the consortia that have evolved in the four atrazine
107 containing environments (Fig 3) and compared them to the ancestral ones. Interestingly, we
108 observed a significant increase of the mineralization potential of the ethylamino chain in evolved
109 consortia compared to ancestors in all but the three nitrogen sources supplemented medium. The

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110 observed gain is however much stronger in the atrazine only supplemented medium (from 48 % ; 111 $CI_{95\%}$=[41.9 – 54.1] to 71.4 % ; $CI_{95\%}$=[69.4 – 73.4]). When evaluating the mineralization potential 112 of the *s*-triazinic cycle, results are quite contrasted with a significant increase of the mineralization 113 potential in the atrazine only supplemented medium (from 48.3 % ; $CI_{95\%}$=[36.5 – 60] to 86.9 %; 114 $CI_{95\%}$=[81.6 – 92.2]), and significant decreases or no change in the two and three nitrogen sources 115 supplemented media. Altogether, these results indicate that the upper part of the atrazine 116 degradation pathway (*atzA*, *trzN*, *atzB* and *atzC*) has been improved in the evolved consortia, 117 presumably leading to increased intermediary metabolite production, such as aminoethanol, 118 ethylamine or hypoxanthine[18]. While the upper pathway is constitutively expressed, studies have 119 shown that the lower pathway was repressed by the presence of ammonium in the environment[23]. It 120 is therefore quite likely that the stronger accumulation of intermediary metabolites and their 121 consumption by consortium members led to an increased concentration of ammonium in the 122 environment explaining the decreased mineralization potential of the *s*-triazinic cycle in evolved 123 consortia compared to ancestors.

124 The impressive boost of atrazine mineralization potential observed in the medium supplemented 125 only with atrazine led us to focus on phenotypic and genotypic changes that occurred in this specific 126 environment. We therefore isolated members of the evolved consortia, evaluated individually their 127 genetic repertoire using multiplex PCRs, and assessed their corresponding growth characteristics 128 using Bioscreen™ assays. We found that only *Arthrobacter* sp. TES was able to grow in isolated 129 culture, while the three other members of the evolved consortia depend on *Arthrobacter* sp. TES to 130 grow in this medium (Fig 4A). This acquired dependency for *Chelatobacter* sp. SR38 and 131 *Variovorax* sp. 38R is explained by losses of part of their *atz* genes repertoire (Fig 4B). 132 *Chelatobacter* sp. SR38 lost part of the upper pathway via *atzA* and *atzB* removal, while *Variovorax* 133 sp. 38R kept only *atzA*, responsible for dechlorination of atrazine which does not provide any 134 nitrogen containing by-product. We then wanted to characterize the interactions between members

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135 of the evolved consortia. We therefore reconstructed all possible duos and trios, and compared
136 growth performances of each member to their performance in the evolved four-species consortia
137 and in monoculture over 3.5 days (Extended Data Fig 1). A relatively simple and straightforward
138 interaction scheme can be drawn from these results (Fig 4C): *Variovorax* sp. 38R, *Chelatobacter* sp.
139 SR38 and *Pseudomonas* sp. ADP growths are clearly supported by *Arthrobacter* sp. TES. However,
140 *Arthrobacter* sp. TES is penalized in duos or trios with *Chelatobacter* sp. SR38 and *Pseudomonas*
141 sp. ADP, in the absence of *Variovorax* sp. 38R. Interestingly, *Variovorax* sp. 38R by itself has no
142 positive effect on *Arthrobacter* sp. TES, but it counteracts the negative impacts of the two other
143 consortium members on *Arthrobacter* sp. TES. Therefore, coexistence of the four members might
144 be favoured, even though two of the three direct beneficiaries exert antagonistic relationships
145 against the public goods provider, because the third one acts as a buffering intermediary.
146 Here, we have witnessed the establishment of dependencies in a four species artificial consortium
147 after evolution in an unstructured, atrazine only supplemented medium during ~100 generations.
148 According to the BQH, dependency was set up *via* adaptive gene loss, and one specific member of
149 the consortium, *Arthrobacter* sp. TES ensuring the costly part of the function, became the provider
150 of public goods to the three other beneficiaries, *Variovorax* sp. 38R, *Chelatobacter* sp. SR38 and
151 *Pseudomonas* sp. ADP. In many cases, pesticide degradation in nature is thought to occur through
152 the involvement of microbial consortia, rather than being the corollary of a single species[4]. Our
153 study provides strong experimental evidences, using the herbicide atrazine as a case study, that the
154 division of labour between populations in microbial communities might be the rule when
155 considering the biodegradation of xenobiotics.

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156 **METHODS**

157 ***Experimental Evolution***

158 Four strains with variable atrazine degrading abilities (*Pseudomonas* sp. ADPe, *Chelatobacter* sp.
159 SR38, *Arthrobacter* sp. TES and *Variovorax* sp. 38R, Fig. 1) were cultivated in seven media in four
160 species consortia (n=3) constituting "community" evolution lines. Mineral salt (MS) media
161 (K_2HPO_4 9.2 mM, KH_2PO_4 3.0 mM, $CaCl_2$ 0.2 mM, $MgSO_4$-7H_2O 0.8 mM, NaCl 1.7 mM, H_3BO_3
162 32.3 µM, $FeSO_4$-6 H_2O 19.2 µM, $MnSO_4$-H_2O 10.6 µM, NaMo 2.1 µM, $ZnSO_4$ 1.2 µM, $CuSO_4$
163 0.63 µM, biotin 0.4 µM, thiamin 0.15 µM,) containing citrate as carbon source (5 mM) were
164 supplemented with varied nitrogen sources combinations (atrazine, cyanuric acid, $(NH_4)_2SO_4$, or 2
165 and 3 sources combinations) with a final nitrogen equimolarity of 1.4 mM (7 media). The evolution
166 experiment was realized in 96-wells 2 mL Deepwell microplate at 28°C without agitation during 23
167 growing cycles of 3.5 days each. For each new cycle, 35 µL of previous culture was used as
168 inoculum for the next cycle into 700 µL fresh medium (1/20 dilution, (log2(20) x 23 cycles ~ 100
169 generations)). An aliquot of each cycle culture was mixed with glycerol (30% final concentration)
170 and preserved at -80°C.

171 ***Species composition***

172 We were able to easily identify the four strains in the ancestor and evolved "community" lines
173 because each of them was double-resistant to a different combination of two of the following
174 antibiotics: rifampicin 100mg/L, kanamycin 50mg/L, spectinomycin 100 mg/l, streptomycin
175 100mg/L. To do so, each bacterial strain was grown in MS medium containing either atrazine or
176 cyanuric acid as the nitrogen source and then spread on solid rich medium (TY) containing the
177 antibiotic for which it was to be resistant. Resistant growing colonies were collected and cultivated
178 in their specific liquid MS medium to ensure that they have kept their metabolic ability towards
179 atrazine. This procedure was repeated to confer a second resistance to each strain. *Pseudomonas* sp.

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180 ADPe is Strep$^+$/Rif$^+$, *Chelatobacter* sp. SR38 is Kan$^+$/Strep$^+$, *Arthrobacter* sp. TES is Spec$^+$/Rif$^+$
181 and *Variovorax* sp. 38R is Spec$^+$/Kan$^+$.
182 Ten-fold successive dilutions of T1 and T23 "community" lines were done and inoculated onto LB
183 agar plates supplemented with different antibiotic combinations. After 3-days of growth at 28°C,
184 CFU were counted across 3 dilution levels.

185 ***Atrazine-degrading genetic repertoire***

186 Multiplex PCR targeting *atzA*/*atzD*/*trzD* and *atzC*/*trzN*/*atzB* genes were used to determine the
187 atrazine degradation repertoire of each strain of the ancestral and evolved "community" lines. 3 or 4
188 isolated colonies from the ancestral and evolved "community" lines were resuspended in water and
189 served as DNA template for PCR reactions. PCR reactions were conducted using the following
190 primers [atzA (5'- TGA AGC GTC CAC ATT ACC-3', 5'- CCA TGT GAA CCA GAT CCT-3'),
191 atzD (5'- GGG TCT CGA GGA TTT GAT TG-3', 5'-TCC CAC CTG ACA TCA CAA AC-3')
192 trzD5'- CCT CGC GTT CAA GGT CTA CT-3', 5'-TCG AAG CGA TAA CTG CAT TG-3'] or
193 [trzN (5'-CAC CAG CAC CTG TAC GAA GG-3', 5'-GAT TCG AAC CAT TCC AAA CG-3'),
194 atzB (5'- CAC CAC TGT GCT GTG GTA GA-3', 5'-AGG GTG TTG AGG TGG TGA AC-3'),
195 atzC (5'- GTACCATATCACCGTTGCCA-3', 5'-GCTCACATGCAGGTACTCCA-3')] at a final
196 concentration of 10 µM each, the temperature of primers annealing being of 57°C and the
197 elongation time of 1 minute.

198 ***Atrazine mineralisation activity***

199 Ancestors and "community" lines that have evolved on the four media containing atrazine were
200 inoculated at ~0.001 OD_{600nm} in 200 µL fresh medium supplemented with 4000 dpm final of ^{14}C
201 atrazine labelled either on the ethylamino chain or on the *s*-triazine cycle in triplicate. Cultures were
202 then placed at 28°C. A whatman® 3mm Chr paper soaked with barite (saturated solution 0.37M)
203 was placed and sealed on the top of the 96-wells plates and replaced periodically during 3.5 days.
204 Dried papers were fluorography printed on Storage PhosphorScreen (Molecular Dynamics®) for 2

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205 days. Screens were scanned by a phosphorimager (Storm 860 Molecular Imager). Indirect reading
206 of released radioactivity intensity was done by ImageQuant 5.2 software, and referred to a standard
207 curve. Mineralisation percentages of lines were determinate with respect to initial radioactivity
208 dosage realized on 200 µL of each culture medium with a Beckman® liquid scintillation counter.

209 ***Population dynamics characteristics of the ancestral and evolved line***

210 The population dynamics of ancestral and evolved "community" lines were measured using
211 Bioscreen™. All lines were inoculated at ~0.001 OD_{600nm} in 400 µL of their evolution medium (n=3
212 for each replicate of each line) in Bioscreen ™ plates. Plates were incubated 3.5 days at 28°C and
213 the evolution of OD_{600nm} was monitiored every 10 minutes after a 10-seconds gentle shake.
214 Population dynamics characteristics of each strain that has evolved in "community" lines was also
215 measured the same way starting from 10 CFU isolated from the evolved "community" lines as
216 inoculums and inoculated at ~0,001 OD_{600nm}. Those data were used to determine the maximal
217 OD_{600nm}. Cultures that did not reach 0.1 OD_{600nm} at the end of the 3.5 days were considered as non-
218 growing.

219 ***Evaluating interactions between the four evolved species in the atrazine only supplemented***
220 ***medium***

221 For each strain, ten CFU isolated from each community-evolved lines (n=3) in MS-Citrate Atrazine
222 were used as inoculum pools. 4-species consortia, as well as all possible duos and trios were
223 reconstructed and compared to monocultures. Mixtures and monocultures were inoculated at ~0,001
224 OD_{600nm} and final population densities, after a 3.5 days cycle, were measured for each strain by CFU
225 plating on TY medium supplemented with the appropriate antibiotics combinations.

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287 **ACKNOWLEDGEMENTS**
288
289 We thank L. Philippot for comments and discussion. Grants from FP7-PEOPLE-2012-IAPP
290 (Industry-Academia Partnerships and Pathways) Marie-Curie project 'Love-to-Hate' funded by the
291 European Commission (Grant Agreement number 389 324349) supported this work.

292

293 **AUTHOR CONTRIBUTIONS**
294
295 LB: laboratory experiments, data analysis, manuscript writing. MD: study conception, laboratory
296 experiments, manuscript editing. NR: laboratory experiments. FM-L: study conception, manuscript
297 editing. AS: study conception, data analysis, manuscript writing.

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304 **FIGURE LEGENDS**

305

306 **Figure 1. Atrazine metabolic degradation pathway and strains used. a,** intermediary products of
307 the upper and lower parts of the metabolic degradation of atrazine, as well as genes associated with
308 the different reactions are indicated. **b,** the four ancestral strains, as well as their corresponding
309 atrazine degrading gene repertoire are indicated. Genes encoding enzymes involved in the
310 formation of N-containing by-products are coloured in blue while genes encoding enzymes
311 involved in the formation of C-containing by-products are contoured.

312

313 **Figure 2. Community composition of the ancestral and evolved consortia across the seven**
314 **selection media. a,** species composition of the ancestral and evolved consortia across the seven
315 selection media. Abundances are expressed in log10 CFU.mL^{-1}. Black, red, green and blue bars
316 correspond respectively to *Variovorax* sp. 38R, *Pseudomonas sp.* ADPe, *Chelatobacter* sp. SR38
317 and *Arthrobacter* sp. TES. *atz*, *nh4* and *cya* respectively stands for nitrogen source used either
318 atrazine, ammonium or cyanuric acid. **b,** atrazine-degrading genetic potential of the ancestral and
319 evolved consortia across the seven selection medium evaluated *via* multiplex PCRs.
320 Presence/absence of the corresponding genes is indicated with the presence/absence of the
321 corresponding coloured or hatched rectangle on the model scheme.

322

323 **Figure 3. Atrazine mineralization potential of the ancestral and evolved consortia.** The
324 mineralization potentials of the ethylamino chain **(a)** and the s-triazinic cycle **(b)** were evaluated
325 with ^{14}C-labelled atrazine across the four atrazine-containing selection media. ^{14}C-labelled atoms
326 are indicated in red on the atrazine molecule. *atz*, *nh4* and *cya* respectively stands for atrazine,
327 ammonium and cyanuric acid. Significant differences between ancestral (dark grey bars) and
328 evolved (light grey bars) consortia performances are indicated with a star ($p < 0.05$).

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330 **Figure 4. Interspecies dependency for atrazine degradation. a,** growth performances of the
331 ancestral (grey bars) and community-evolved (black bars) strains estimated as the final OD_{600nm}
332 reached after a 3.5 days cycle. Strains were considered as non-growing if their final OD_{600nm} was <
333 0.1 Arbitrary Units (AU). **b,** atrazine-degrading genetic repertoire of the community-evolved strains
334 and corresponding metabolic reactions. **c,** interactions scheme drawn from mono- and co-cultures of
335 the four community-evolved strains in the atrazine only supplemented medium. Arrows represent
336 either positive (+) or negative (-) interactions. The dotted arrow expresses the conditional positive
337 effect of *Variovorax* sp. 38R on *Arthrobacter* sp. TES in the presence of the two other strains.

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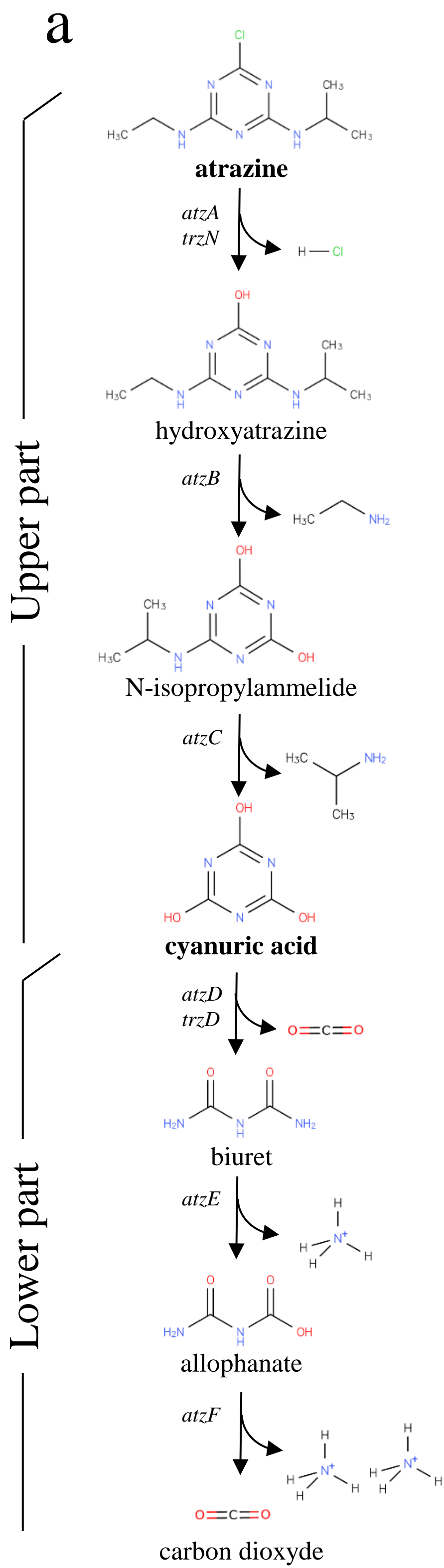
338 **EXTENDED DATA LEGENDS**

339

340 **Extended Data Figure 1.** Evaluating interactions between the four species evolved in the atrazine
341 only supplemented medium. Growth performances of evolved *Variovorax* sp. 38R (**a**), *Arthrobacter*
342 sp. TES (**b**), *Chelatobacter* sp. SR38 (**c**) and *Pseudomonas* sp. ADPe (**d**) at the end of a 3.5 days
343 cycle in monoculture, as well as in reconstructed duos, trios and in the 4-species consortium were
344 evaluated using CFU plating on TY medium supplemented with the adequate antibiotics
345 combinations. Bars represent mean values with s.e.m (n=3).


a
Upper part
Lower part
atrazine
atzA
trzN
hydroxyatrazine
atzB
N-isopropylammelide
atzC
cyanuric acid
atzD
trzD
biuret
atzE
allophanate
atzF
carbon dioxyde
b
Variovorax 38R
Arthrobacter TES
Chelatobacter SR38
Pseudomonas ADPe
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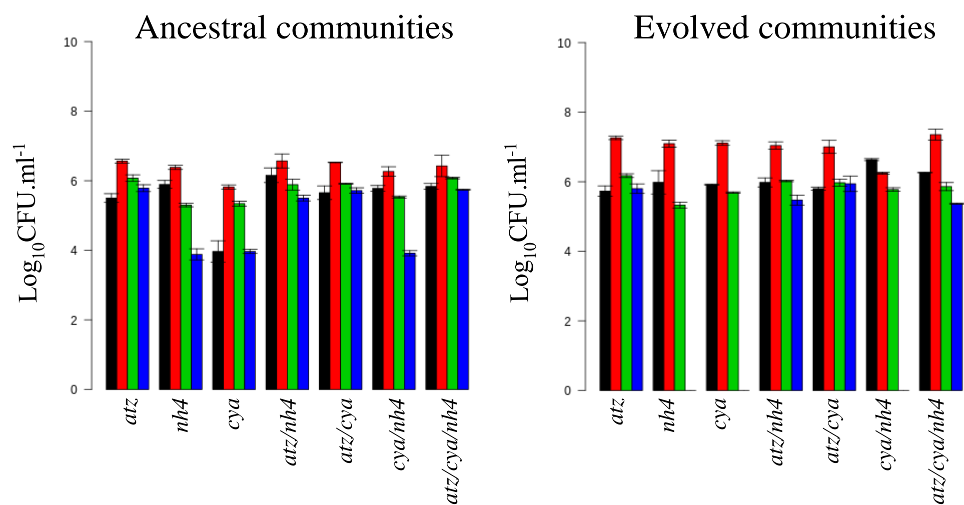

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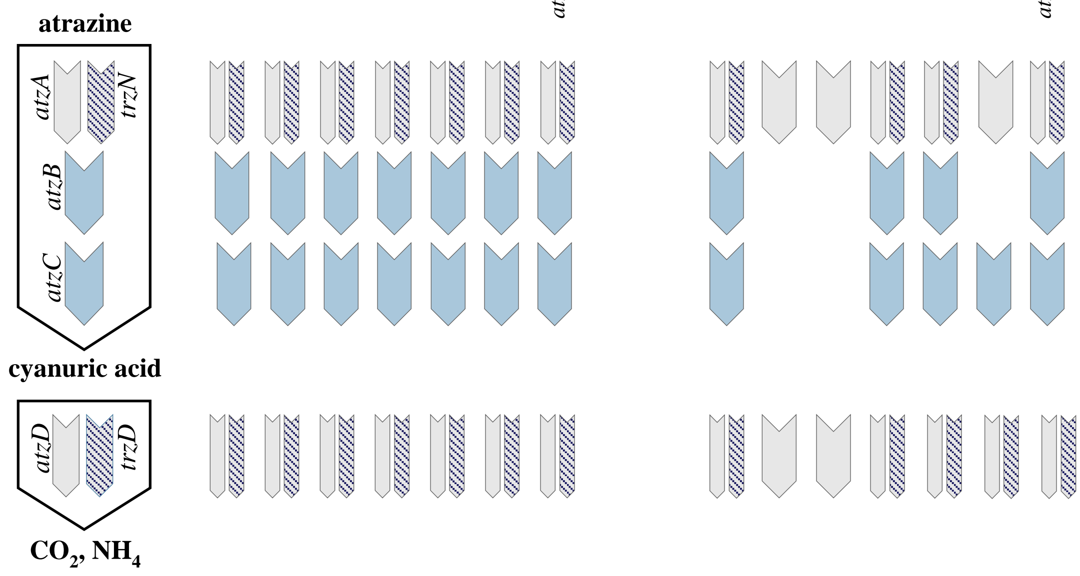
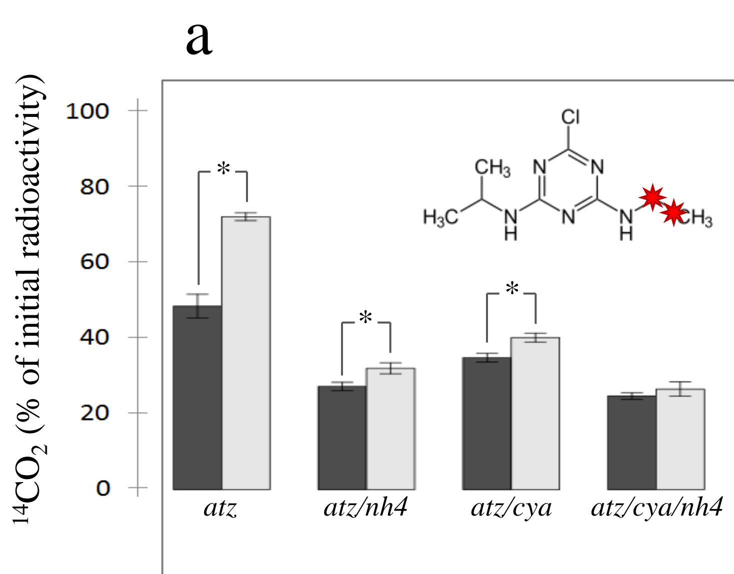
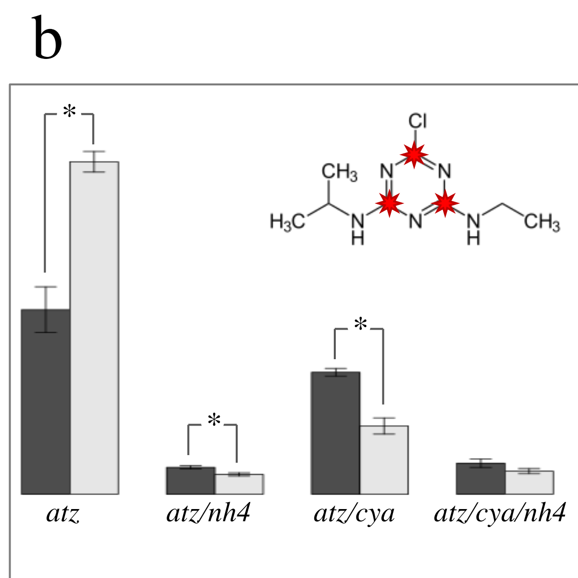



a
$^{14}CO_2$ (% of initial radioactivity)
100
80
60
40
20
0
*
Cl
CH3
N
N
H3C
N
H
N
N
H
CH3
atz
atz/nh4
atz/cya
atz/cya/nh4


b
*
Cl
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atz
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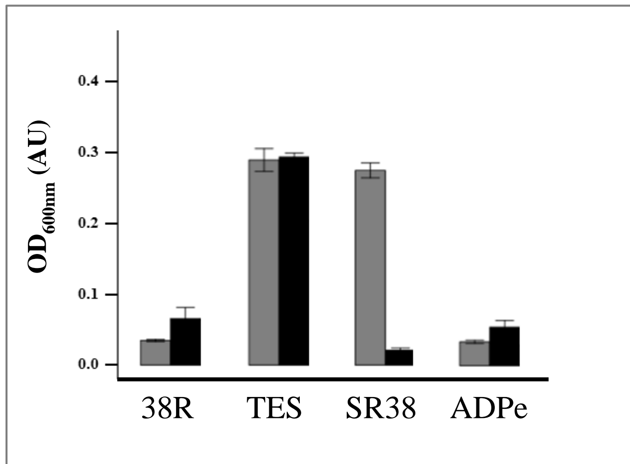
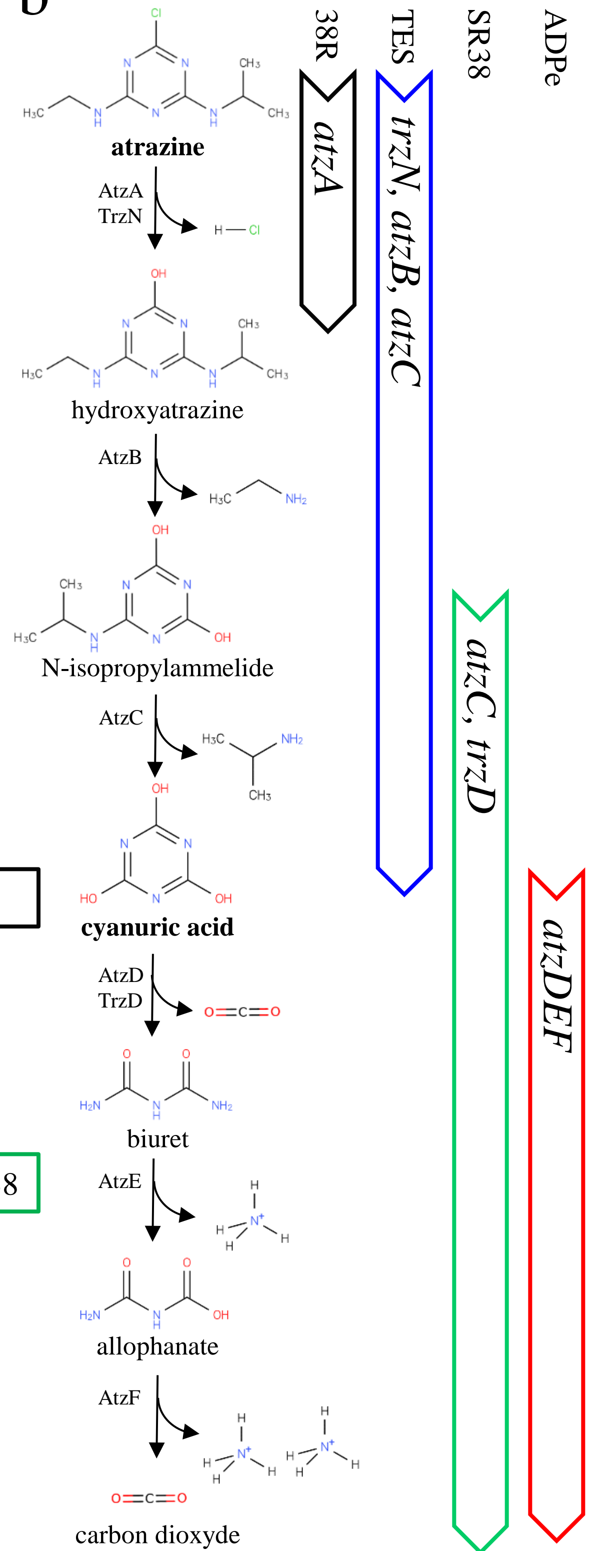


b



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