

1 **Dual-stressor selection alters eco-evolutionary dynamics in**
2 **experimental communities**

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23
24 **Recognizing when and how rapid evolution drives ecological change is fundamental for**
25 **our understanding of almost all ecological and evolutionary processes such as community**
26 **assembly, genetic diversification and the stability of communities and ecosystems.**
27 **Generally, rapid evolutionary change is driven through selection on genetic variation as**
28 **well as affected by evolutionary constraints such as trade-offs and pleiotropic effects, all**

29 contributing to the overall rate of evolutionary change. Each of these processes can be
30 influenced by the presence of multiple environmental stressors reducing a population's
31 reproductive output. Potential consequences of multi-stressor selection for the
32 occurrence and strength of the link from rapid evolution to ecological change are unclear.
33 However, understanding these is required for predicting when rapid evolution might
34 drive ecological change. Here we investigate how the presence of two stressors affects this
35 link using experimental evolution with the bacterium *Pseudomonas fluorescens* and its
36 predator *Tetrahymena thermophila*. We show that the combination of predation and
37 sublethal antibiotic concentrations delays the evolution of anti-predator defence and
38 antibiotic resistance compared to the presence of only one of the two stressors. Rapid
39 defence evolution drove stabilization of the predator-prey dynamics but this link between
40 evolution and ecology was weaker in the two-stressor environment, where defence
41 evolution was slower, leading to less stable population dynamics. Tracking the molecular
42 evolution of whole populations over time showed further that mutations in different genes
43 were favoured under multi-stressor selection. Overall, we show that selection by multiple
44 stressors can significantly alter eco-evolutionary dynamics and their predictability.

45 Microbes often adapt surprisingly fast to changes in their environment. For instance, the rapid
46 adaptation of resistance against pesticides or antibiotics^{1,2}, as well as the coevolution of
47 interacting microbes³⁻⁵, suggest an abundant supply of adaptive genetic variation. It is now well
48 established that the dynamics of rapid evolutionary change can determine the ecological
49 dynamics of populations and communities, which can again alter further evolutionary change
50 and so on⁶⁻⁸. Because microbial communities determine the functioning of nearly all
51 ecosystems⁹, understanding their eco-evolutionary dynamics is of fundamental importance, for
52 example, for predicting harmful bacterial blooms¹⁰, the community composition of the

53 holobiont¹¹ or the potential of a microbial community to serve as a reservoir for antibiotic
54 resistance alleles².

55 Recent work has uncovered important consequences of eco-evolutionary dynamics, for
56 example, for the coexistence of interacting species¹², temporal changes in their population
57 sizes⁶ and the maintenance of diversity^{3,13}. Eco-evolutionary dynamics and their consequences
58 are typically studied in the presence of one environmental stressor that leads to a reduction in
59 fitness (e.g. one consumer or the exposure to antibiotics). However, the underlying mechanisms
60 linking evolutionary and ecological change are virtually unknown in communities with more
61 than one stressor (e.g. consumer and antibiotics). Previous work has examined multi-stressor
62 selection^{14,15}, but this work has been limited to investigations of the evolutionary or ecological
63 dynamics rather than the links between ecology and rapid evolution. One important question
64 with multiple stressors is whether or not the same links between evolution and ecology matter
65 as with one stressor. Here we develop predictions for the link between the evolutionary and
66 ecological dynamics in single and multiple stressor environments and test these in an
67 experimental evolution study. We focus on two commonplace stressors in microbial
68 communities—ciliate predation and sublethal antibiotic concentrations (sub-minimum
69 inhibitory concentrations; hereafter, sub-MICs)—and disentangle for the first time key
70 processes driving the link between ecological and evolutionary dynamics in bacteria-ciliate
71 communities.

72 Sub-MIC levels are commonly found e.g. in sewage waters, lakes, rivers and soil¹⁷, and
73 they have been shown to select for antibiotic resistance either by an increase in the frequency
74 of resistant bacteria or by selection for *de novo* resistance¹. Besides evolutionary consequences,
75 sub-MICs of antibiotics can also affect ecological dynamics, for example, by lowering bacterial
76 population sizes when sub-MICs of antibiotics do not alter growth rates but increase density
77 independent mortality rates². From these observations, we can further predict a direct link

78 between evolutionary and ecological dynamics when resistance evolution leads to higher
79 growth rates or compensation for increased death rates in the presence of sub-MICs such that
80 bacteria reach similar densities as without sub-MICs.

81 Consumption by protists or phages exerts strong selection on the bacterial prey
82 population apart from the ecological effect of driving bacterial population. Bacteria are known
83 to rapidly evolve anti-predatory adaptations against consumers, e.g. by evolving to grow in
84 colonies or as biofilm, thereby decreasing attack rates or increasing handling time^{16,17}. General
85 ecological theory for predator-prey interactions predicts that decreasing attack rates and/or
86 large increases in handling time can result in stabilization of the temporal dynamics of the prey
87 and its consumer¹⁸, which can be seen in oscillations with reduced amplitudes or a shift to
88 steady state dynamics (Supplementary Information Fig. S1; note that the conditions for stability
89 depend on the details of the model applied, e.g., the functional response of the predator)¹⁹. Thus
90 the evolution of defence traits can directly affect the ecological dynamics within predator-prey
91 systems, which has been confirmed in models²⁰ and experiments²¹⁻²³.

92 Sub-MIC levels of antibiotics can, however, alter the evolution of anti-consumer defence
93 traits in bacterial populations^{4,24}, and the presence of the two stressors also has the potential to
94 alter the stability of the microbial community (ecology). Multi-stressor selection can prevent
95 or delay the evolution of resistance and/or anti-predatory defences through lowering selection
96 strength on individual loci¹⁴, clonal interference where adaptive mutations compete for fixation
97 in large asexual populations²⁵, trade-offs between traits^{26,27}, pleiotropy²⁸ or linkage
98 disequilibrium²⁹. Furthermore, the evolution of one trait can alter the strength of species
99 interactions and thus selection, which can lead to slower evolution, or favour different
100 mutations due to differences in associated costs, or because the role of the order of mutations
101 changes in different environments. Bacterial population sizes are predicted to be lower in the
102 presence of the two stressors as the combined effect lowers fitness even more, which can affect

103 evolutionary change by reducing mutation supply and increasing the relative importance of
104 drift to selection^{30,31}. Alternatively, pleiotropic effects of mutations might accelerate the
105 evolution of one trait when adaptation to one stressor provides adaptation to the second one at the
106 same time. The effects of clonal interference could be alleviated in small population sizes, as
107 clonal interference occurs less often when mutation supply is low^{32,33}. The pace of evolution is
108 also predicted to be faster when the predator removes selectively maladapted individuals or
109 through the evolutionary hydra effect³⁴. Finally, we predict that differences in the rate of
110 evolution impact the population dynamics of the bacterial prey and the predator, with slower
111 evolution leading to less stable and faster evolution to more stable predator-prey dynamics
112 under the assumption of stabilizing selection³⁵.

113 To test for the role of multi-stressor selection for eco-evolutionary dynamics, we exposed
114 in an experimental evolution study initially isogenic populations of the bacterium
115 *Pseudomonas fluorescens* SBW25 to 0×MIC and 0.1×MIC of the antibiotic streptomycin
116 (mode of action: inhibition of protein synthesis in prokaryotes) in the presence and absence of
117 the ciliate *Tetrahymena thermophila* in a full-factorial experiment for ~220 bacterial and ciliate
118 generations (66 days; Material and Methods). We followed population dynamics and
119 phenotypic changes of three replicated populations in each treatment. To gain mechanistic
120 insights into how sub-MICs and predation altered the evolution of defence and resistance, we
121 analysed whole-genome sequence data from the replicate bacterial populations over time. This
122 allowed us to compare when *de novo* mutations (single nucleotide polymorphisms, SNPs;
123 insertions or deletions, INDELs; copy number variations, CNVs) arise and their dynamics over
124 time across the different treatments.

125 **Results & Discussion**

126 We observed different ecological and evolutionary dynamics over time depending on the
127 presence/absence of the ciliates, as well as between treatments with the presence of

128 streptomycin (Fig. 1). Streptomycin did not have a direct effect on the maximum growth rates
129 of ciliates and bacteria (Fig. S2). However, bacterial densities were significantly lower with
130 streptomycin (Generalized Estimating Equations model (GEE) bacteria alone: sub-MIC:
131 $W=19.11$, $df=1$, $p=1.236 \cdot 10^{-5}$; for all non-significant results, see Supplementary Information;
132 Table S1) as well as in the presence of ciliates (GEE: interaction sub-MIC \times day: $W=4.96$, $df=1$,
133 $p=0.026$; day: $W=29.54$, $df=1$, $p=5.47 \cdot 10^{-8}$; sub-MIC: $W=61.68$, $df=1$, $p=3.997 \cdot 10^{-15}$). Overall,
134 population dynamics were less stable in the presence of the ciliates and even less in the
135 presence of ciliates with streptomycin (de-trended standard variation of the predator
136 population=coefficient of variation: Generalized linear model (glm): $F=16.963$, $df=2$,
137 $p=0.0146$; Figs. 1, 2a). Bacteria-ciliate populations showed considerable fluctuations at the
138 beginning of the experiment in the presence and absence of streptomycin, but stabilized in the
139 latter case around day 25. In the predator-free treatments, bacterial population sizes showed
140 only small fluctuations around the carrying capacity (Fig. 1a,b). Thus, the sub-MIC and the
141 presence of the predator led to lower bacterial population sizes and the predator to less stable
142 dynamics, which was stronger with sub-MIC streptomycin.

143 To follow the evolutionary response of predation by the ciliate, we measured growth
144 rates r of the ancestral predator when growing on ancestral and evolved bacteria isolated from
145 different time points of the experiment. From this we calculated the defence level $D =$
146 $\left(1 - \frac{r_{\text{evolved}}}{r_{\text{ancestor}}}\right)$ with 0 meaning that the evolved bacteria have the same level of defence as the
147 ancestor and values close to 1 a very high level of defence compared to the ancestor¹⁶. Bacteria
148 evolved anti-predatory defence by forming biofilm and/or colonies (Fig. S3), with significantly
149 higher levels of defence with 0 \times MIC levels over time (GEE: day: $W=13.03$, $df=1$, $p=0.00031$;
150 sub-MIC: $W=15.38$, $df=1$, $p=8.81 \cdot 10^{-5}$; Fig. 1b, d; Table S2). Lower predator growth rates were
151 attributed to significantly lower ingestion rates for the defended prey compared to the
152 undefended ancestral prey (ANOVA starting concentrations vs. ingestion: interaction

153 concentration×defence: $F_{1,60}=11.44$, $p=0.001$; concentration: $F_{1,60}=76.67$, $p=7\cdot 10^{-12}$; defence:
154 $F_{1,60}=20.86$, $p=2.51\cdot 10^{-5}$; Fig. S4), which could be the result of lower attack rates or increased
155 handling times (Fig. S1).

156 We found the evolution of streptomycin resistance in populations in the predator-free
157 environments with $0.1\times\text{MIC}$ (Fig. 1c), which we confirmed by testing individual isolates from
158 the end of the experiment (glm for the comparison ancestor vs. isolates from the end of the
159 experiment: $F=37.6$, $df=8$, $p=4.6\cdot 10^{-5}$; Fig. S5). Importantly, however, streptomycin resistance
160 was not observed in the $0.1\times\text{MIC}$ populations with predators (glm for the comparison ancestor
161 vs. isolates from the end of the experiment with family: $F=2.32$, $df=8$, $p=0.15$; Figs. 1d, Fig.
162 S5). To test whether resistance evolution was delayed or not occurring, we followed the
163 frequency of resistance evolution in 48 additional populations with a factorial design including
164 the presence and absence of ciliates in $0.1\times\text{MIC}$ streptomycin (Material and Methods). We
165 found an increase in the frequency of resistant populations within 16 days in all treatments but
166 the overall level of resistance was lower in the presence of the ciliates in $0.1\times\text{MIC}$ (GEE:
167 interaction day×treatment: $W=35.46$, $df=3$, $p=9.738\cdot 10^{-8}$; treatment: $W=440.5$, $df=1$, $p<2.2\cdot 10^{-}$
168 16 ; day: $W=14.15$, $df=1$, $p=0.00014$; Fig. S6). Thus, resistance and defence evolution were
169 delayed in the presence of the two stressors.

170 Next, we investigated the links between the ecological and evolutionary dynamics over
171 time and across different environments. Bacterial population sizes were significantly lower in
172 the presence of $0.1\times\text{MIC}$ streptomycin even after they evolved resistance (Fig. 1a,c) suggesting
173 that the evolution of resistance had no effect on the ecological dynamics of the system. We
174 found a significant negative correlation between stability of the bacteria-ciliate communities
175 and defence level (glm: mean defence level: $F=21.96$, $df=1$, $p=0.0094$; Fig. 2b) suggesting that
176 the evolution of defence altered the predator-prey dynamics as predicted by ecological theory
177 (Fig. S1). To further test whether the degree of defence alters the stability of the predator-prey

178 system, we repeated the experiment but starting with clonal bacterial lineages differing in their
179 defence level in the presence and absence of 0.1×MIC streptomycin (Material and Methods).
180 Again, we found a significant negative correlation between stability and defence level but
181 independent of the presence of sub-MIC levels of streptomycin (glm: level of defence of initial
182 clone: $F=14.06$, $df=1$, $p=0.00057$; Fig. 2c). Thus the evolution of defence altered the ecological
183 dynamics of predator and prey and, importantly, the presence of sub-MIC streptomycin did not
184 directly affect the predator-prey dynamics but rather indirectly by slowing down defence
185 evolution. Thus the relative role of defence evolution for the predator growth was lower in the
186 presence of sub-MIC streptomycin compared to the ecological change, i.e., the number of
187 available prey (ratio evolutionary change: ecological change: 0×MIC: 1.2 ± 0.5 and 0.1×MIC:
188 0.28 ± 0.3 ; ANOVA: $F=8.72$, $df=1$, $p=0.042$; following the Geber method described in^{16,36}; Fig.
189 S7).

190 We confirmed this in additional experiments using two antibiotics with different modes
191 of action at 0.1×MIC (rifampicin: inhibition of RNA synthesis; tetracycline: inhibition of
192 protein synthesis). Bacteria evolved anti-predator defence very rapidly with tetracycline but
193 not with rifampicin. Also in these cases bacterial population sizes were lower in the antibiotic
194 treatment without predator, and defence evolution affected the stability of predator-prey
195 dynamics with lower stability in the absence of defence evolution (Fig. S8).

196 Our data show that the combination of sub-MIC levels of streptomycin and predation
197 slowed down the evolution of anti-predator defence as well as antibiotic resistance. Clonal
198 interference, differences in mutation supply, genomic constraints such as epistatic interactions
199 and pleiotropic effects, and differences in the strength and directionality of selection could
200 explain these observations^{14,25,27,37-39}. With clonal interference, we would predict to find
201 subpopulations of clones that are either resistant against streptomycin or defended against
202 ciliates, but not both. We did not find evidence for this when estimating correlations between

203 these two traits in bacterial clones from populations with 0.1×MIC streptomycin and ciliates
204 isolated from the end of the experiment (Fig. 3a). We rather found a significant positive
205 correlation indicating a pleiotropic effect for defence and resistance (glm: logMIC~ defence
206 level: $F=36.5$, $df=1$, $p=1.2 \cdot 10^{-7}$; Table S3). Such an effect was absent in the populations
207 evolving in the presence of only the ciliates (glm: logMIC~ defence level: $F=0.59$, $df=1$,
208 $p=0.44$; Fig. 3a). There were also no costs associated with defence and MIC levels of individual
209 clones that could hinder the evolution of resistance or defence as we observed only positive
210 correlations with maximum growth rates when tested in the absence of either of the stressor
211 from the 0.1×MIC streptomycin and ciliate populations (glm: $r_{\max} \sim \log\text{MIC}$: $F=43.6$, $df=1$,
212 $p=1.5 \cdot 10^{-8}$; $r_{\max} \sim$ defence level: $F=30.7$, $df=1$, $p=8.1 \cdot 10^{-7}$), which were absent in the one
213 stressor environments (Fig. 3b,c; Table S3).

214 To further investigate the mechanisms slowing down evolution of defence and resistance,
215 we used whole-genome sequence data from the replicate bacterial populations over time. For
216 this, we isolated DNA from subsamples of the populations at 10 time points (Material and
217 Methods; Supplementary Information). We applied a pipeline to distinguish mutations from
218 sequence errors and identified CNVs, short variants (SNPs, INDELs) and cohorts of variants
219 with similar dynamics over time. We found a large number of variants in all populations (Figs.
220 4a, S9) likely because bacterial populations were not mutation-limited (average size $>10^8$
221 individuals), which also suggests that the role of drift was negligible in our populations.
222 However, the number of variants differed significantly (glm: $\chi^2=3393$, $df=8$, $p<2.2 \cdot 10^{-16}$; Fig.
223 4a), with most mutations in the populations where bacteria evolved in the presence of 0.1×MIC
224 and fewest in the presence of the ciliate (Table S4). These differences in the overall number of
225 mutations are likely explained by the evolution of mutator lineages in some replicates (Fig.
226 4a). The majority of mutations were synonymous substitutions (Fig. 4b) and there were no
227 differences between the treatments in the fraction of synonymous mutations (glm with

228 proportion data: $\chi^2=84.8$, $df=8$, $p=0.33$), suggesting that there were no or only little differences
229 in mutation supply.

230 In further analyses, we focused on genes involved in antibiotic resistance and associated
231 with the wrinkly spreader colony phenotype⁴⁰ and derived variants within genes that reached a
232 frequency in at least one population of 50% (hereafter filtered variants). We focus on the
233 wrinkly spreader phenotype as it has previously been shown to be selected by predation (the
234 phenotype forms biofilm)¹⁷ and we found a higher frequency of wrinkly spreaders in the
235 presence of predation independent of the streptomycin concentration (glm: predation: $F=248.3$,
236 $df=1$, $p=2.627 \cdot 10^{-7}$; Figs. S3, S10, Table S5). The number of selected mutations differed
237 significantly between the different treatments (glm: $\chi^2=74.87$, $df=8$, $p=0.0091$; Fig. 4b) with
238 fewest variants in the environment with $0.1 \times \text{MIC}$ and the ciliates present (glm: predation:
239 $\chi^2=79.24$, $df=10$, $p=0.0076$; antibiotics: $\chi^2=79.18$, $df=9$, $p=0.8168$; predation \times antibiotics:
240 $\chi^2=74.87$, $df=8$, $p=0.038$).

241 We identified one gene related to antibiotic resistance (*rpsL*) in populations exposed to
242 antibiotics alone and where we observed streptomycin resistance evolution (Figs. 1c,4c). The
243 third replicate, where we did not observe streptomycin resistance evolution had no mutation in
244 the known resistance related gene. For the populations exposed only to predation, we found in
245 all three replicate populations a duplication that did not occur in other treatments arising around
246 day 20–30 (Fig. 4c), and in two populations, the fixation of mutations in the gene PFLU 4745
247 (Figs. 1b, 4c). For populations evolving in the presence of antibiotics and the predator, we
248 found different mutations reaching high frequencies. Mutations in the gene *ptsP* were found at
249 high frequency in all three replicate populations and mutations in *wspF* and *gacS* in two
250 replicates. The latter two have previously been associated with the wrinkly spreader
251 phenotype^{41,42}, and *ptsP* has a proposed global regulatory function for gene expression⁴³.

252 Mutations in the same genes in replicate populations can be considered evidence for
253 fitness benefits of these mutations. While we found 1–3 multi-hit genes within treatments (Figs.
254 1,4), we found only one gene with derived variants present in the one and two stressor
255 environments in at least two out of the three replicates (*ptsP*, Fig. 4c) but not in the control
256 populations without any stressor. The lack of overlap across treatments but not across replicates
257 within treatments in derived alleles suggests that different mutations were selected with one or
258 two environmental stressors. Furthermore, in the one-stressor environment, derived alleles
259 swept to high frequencies in all replicates (Fig. 4b,c). In the two-stressor environment, we
260 found sweeps as well as additional sweeps at a later time point and before the preceding sweeps
261 were close to fixation. In one replicate (4d, middle row, replicate 2) mutations that reached
262 high frequencies (*ptsP* and *wspF*) went extinct and were replaced by others, which could either
263 be the result of an additional detrimental mutation in this genetic background or clonal
264 interference. Interestingly, these frequency changes correlate with changes in the predator-prey
265 dynamics (Fig. 1d, top row, replicate 2; a decrease in predator and increase in bacteria densities
266 around day 50), which we did not observe in the other two replicates where the derived allele
267 of *ptsP* stayed at high frequencies. We observed two or several derived alleles with the same
268 trajectories within the same populations in the presence of 0.1×MIC (Fig. 1c,d) indicating
269 genetic hitchhiking, where driver mutations carry along other mutations.

270 Based on general ecological theory for predator-prey systems (Fig. S1), we predicted
271 differences in the eco-evolutionary dynamics of bacteria and ciliate communities in the
272 presence and absence of antibiotic stress through slower evolution of anti-predator defences in
273 the presence of the antibiotics. These differences in the evolutionary dynamics altered the
274 ecological dynamics. Thus, our experiment showed a significant change in the link from
275 evolution to ecology in the presence of both stressors. The slower phenotypic evolution was
276 the result of different mutations increasing to high frequencies in the one and two-stressor

277 environments. Thus the genomic changes driving eco-evolutionary dynamics¹¹ and the link
278 between evolution and ecology might depend on the system and the specific conditions.

279 For the two stressors examined here we can suggest that sub-MIC levels of antibiotics
280 have significant ecological and evolutionary effects on communities and alter the dynamics of
281 the microbial loop as well as its link to ecosystem functioning and nature conservation⁴⁴. Ours
282 and other recent studies examining eco-evolutionary dynamics and multi-stressor
283 selection^{3,12,45} suggest that the type of species interaction and stressor determines the potential
284 mechanism whereby multiple stressors affect the links between ecology and evolution.
285 Consequently, the strength of the link between evolution and ecology depends on other
286 stressors or environmental factors, making predictions on when to find eco-evolutionary
287 dynamics challenging.

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404

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415 **Data Availability Statement:** Data reported in the paper will be archived in a community
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418 konstanz.de.

419 **Competing Interests Statement:** The authors declare no competing financial interests.

420 **Legends:**

421 **Figure 1| Prey (a,c, top), predator-prey (b,d, top), prey defence D (middle), resistance**
422 **(middle) and derived allele frequency (bottom) dynamics from *P. fluorescens* populations**
423 **exposed sub-MIC levels of streptomycin.** Shown are three replicates 1-3 (left to right). **a,b)**
424 **No antibiotics. c,d) 0.1×MIC streptomycin. a,c) Ciliate absent. b,d) Ciliate present.** Bacteria
425 are 10^8 cells/ml (black squares), ciliates are 10^4 cells/ml (blue circles), defence level D (green
426 circles), resistance (logMIC in $\mu\text{g/ml}$, red triangles) and derived alleles are shown in black and
427 different symbols when only found in one population, in orange and with the same symbol
428 when found in more than one population. We only show trait data (defence, log(MIC)) for
429 treatments where they were collected, and when derived alleles passed filtering steps and
430 reached at least 50% frequency in non-mutator populations (Material and Methods). Note that
431 the logMIC values are higher in c and that 10 is the maximum we could measure.

432 **Figure 2| Stability of bacteria-ciliate populations exposed to 0× or 0.1×MIC sub-MIC**
433 **levels of streptomycin and correlation of stability with defence levels of bacteria**
434 **populations. a)** Standard deviations ($\text{SD} \times 10^4$) for de-trended ciliate time series were
435 significantly higher for populations exposed to 0.1×MIC. Symbols: replicates; horizontal bar:
436 mean (corresponds to Figs. 1b,d). **b)** SDs were negatively correlated with the mean levels of
437 defence that evolved over time; Symbols correspond to a. **c)** Significant negative correlation
438 between SDs (SD for de-trended predator densities of the first ten transfers, i.e. before further
439 evolution of defence) and initial defence level of the bacteria at the start of the experiment.
440 Bacteria with different defence levels were grown in the absence of streptomycin (dark grey,
441 circle) and presence of 0.1×MIC streptomycin (light grey, square). For statistical tests, see main
442 text.

443 **Figure 3| Trait correlations of clonal isolates from *P. fluorescens* populations exposed to**
444 **0.1×MIC streptomycin and the ciliate *T. thermophila* from the end of the experiment (day**

445 **66. a)** MIC and defence level *D*. **b)** Maximum growth rate of the bacteria and MIC ($\mu\text{g/ml}$).
446 **c)** Maximum growth rate and *D*. Light grey triangles and regression lines = clones from ciliates
447 + $0.1\times\text{MIC}$, dark grey circles and regression lines from $0\times\text{MIC}$ and ciliates. For statistical tests,
448 see main text.

449 **Figure 4| Molecular evolution of *P. fluorescens* populations exposed to $0.1\times\text{MIC}$**
450 **streptomycin and the ciliate *T. thermophila* in a factorial design. a)** Total number of
451 mutations (SNPs and small INDELS) accumulated over 66 days in *P. fluorescens* in the control
452 populations, in the presence of predation, the presence of $0.1\times\text{MIC}$ streptomycin and in the
453 presence of both. Blocks within the bars represent replicates ($n=3$). The pound key (#)
454 represents the occurrence of a known mutator allele in the population (*mutL* or *mutS* gene). **b)**
455 Number of mutations at high frequencies ($>50\%$) in populations and in genes related to
456 antibiotic resistance and anti-predator defence (see main text). **c)** Genomic variants across
457 replicated populations for 145 genes and 5 large duplications in the *P. fluorescens* SBW25
458 genome. Only variants passing filtration criteria are displayed (total: 190 variants). Heat map
459 colour from white (0.0) through orange (0.5) to red (1.0) indicates the maximum frequency of
460 a SNP or short indel obtained in a population over time (66 day evolutionary experiment). Blue
461 bars indicate the presence of large duplications. Columns represent variable genes or genomic
462 duplications ordered from left to right according to their locus along the genome. Rows
463 numbered 1–3 within treatments represent replicates 1–3.

464 **Material and Methods**

465 **Study system and microcosm experiments:** As a prey species we used the bacterial strain
466 *Pseudomonas fluorescens* SBW25⁴⁶ and as a predator the ciliated protozoan *Tetrahymena*
467 *thermophila* 1630/1U (CCAP). Prior to the experiments, the bacterial stock was kept at -80°C
468 and ciliate stocks were cultured axenically in proteose peptone yeast extract (PPY) medium
469 containing 20 g of proteose peptone and 2.5 g of yeast extract in 1 liter of deionized water. All
470 treatments were started from one clonal culture of bacteria to achieve minimum initial genetic
471 variability in populations. Experiments lasted 66 days, representing approximately 220
472 bacterial and ciliate generations.

473 **Community experiments:** Experiments testing the community dynamics were conducted in
474 standard 25 ml glass vials^{12,16,40,47} with 6 ml medium containing M9 salts and King's B (KB)
475 nutrients at 5% concentration (5% KB: 1 g/l Peptone number 3 and 0.5 ml/l glycerol). Every
476 48 hours, 1% of each culture was transferred to a new vial containing fresh culture medium.
477 Microcosms were kept at $28 \pm 0.1^{\circ}\text{C}$ and shaken constantly at 50 rpm. Population sizes were
478 estimated using optical density measurements and light microscopy counts¹⁶. Evolution of the
479 prey defence trait *D* against predator grazing was quantified with a simple, ecologically
480 appropriate bioassay where growth rates of the predator are measured and compared between
481 ancestral and evolved prey^{12,16}. We used Liofilchem MIC strips to measure antibiotic resistance
482 over time for the evolving populations (Supplementary Information, Fig. 5) and for clonal
483 isolates from day 66. We set up a first experiment adding $0\times$ or $0.1\times$ MIC streptomycin to
484 microcosms of bacteria with and without ciliates with three replicates per treatment (12
485 microcosms in total). A second set of experiments was set up at a later time point using
486 $0.1\times$ MIC of rifampicin and tetracycline in bacterial microcosms with and without ciliates (four
487 replicates each, 16 microcosms in total). We analysed the second set of experiments separately.

488 In order to assay colony phenotype frequencies, we plated diluted samples from day 66 on PPY
489 agar, and categorized the types according to Ref. ⁴⁰.

490 **Evolution of antibiotic resistance:** A second experiment was used to test for the interactive
491 role of predation and a sub-MIC of streptomycin on the evolution of antibiotic resistance. The
492 experiment was conducted in 96-well plates where populations were transferred into fresh
493 culture medium every 48 hours using a pin-replicator⁴⁸ in medium without streptomycin or
494 with $0.1 \times \text{MIC}$ streptomycin and with or without ciliates. Proportions of resistant populations
495 were tested by plating each of the populations onto agar containing an above MIC
496 concentration of streptomycin ($25 \mu\text{g/ml}$). For the analyses, we used differences in the
497 proportion of resistant populations between 0 and $0.1 \times \text{MIC}$ per time point and contrasted these
498 between the ciliate present and absent treatments.

499 **Data analyses:** All statistical analyses were performed in the R statistical environment⁴⁹ using
500 the *lme4*⁵⁰ and the *geepack*⁵¹ packages. Data from the experiments with streptomycin and
501 tetracycline/rifampicin were analysed separately as they were performed separately. We used
502 consumer specific Generalized Estimating Equations models (GEE; bacteria alone or bacteria
503 and ciliate) for the analyses of bacterial and ciliate densities as well as predator-prey ratio and
504 defence level D over time with day and sub-MIC (0 and $0.1 \times \text{MIC}$). We used the family Gamma
505 and the link function inverse for density data and the family Poisson and the link function
506 identity for the D . For the stability analyses of the communities, we calculated the standard
507 deviation of predator population size after de-trending the time-series and scaling the mean to
508 0 using the R package *pracma*⁵². To test for differences in stability between treatments and a
509 relationship between stability and maximum D , we used generalized linear models (glm) with
510 the family Gamma and the link function inverse. Differences in ingestion rates for defended
511 and naive bacteria (Fig. S4) were tested using linear models. The evolution of resistance with
512 and without ciliates in 48 replicate populations (Fig. S6) was compared using generalized linear

513 models with the family Gamma and the link function inverse. For the correlations between D
514 and resistance of the clones from the end of the experiment, we used a generalized linear model
515 with the family Gaussian and the link function identity, and for the correlation between r_{\max}
516 and D as well as r_{\max} and resistance, glms with the family Gamma and the link function inverse.
517 To test for the effect of predation on the frequency of WS evolution, we used glms with the
518 family Gamma and the link function inverse.

519 **Sequence analyses:** Bacterial DNA was extracted (DNeasy Blood & Tissue Kit, Qiagen)
520 directly from 0.5 ml freeze-stored whole-population sample without culturing steps to retain
521 allele frequencies intact. We sequenced the following populations: i) populations without
522 antibiotics or predators (control), ii) with antibiotics (0.1×MIC streptomycin), iii) with
523 predators and iv) with both predators and 0.1×MIC streptomycin. For each treatment, all three
524 replicate populations were sequenced from 10 time points over the course of the 66-day
525 experiment. We focused on early time points, since adaptive mutations were expected to
526 emerge early in rapidly evolving bacterial populations (sequence data generated for days 2, 4,
527 8, 12, 22, 32, 42, 50, 56, 66). Paired-end libraries were prepared using Illumina Nextera XT
528 sequence reads obtained by high-throughput sequencing (Illumina Nextseq 500 high output;
529 for coverage see Table S6).

530 After mapping reads to the reference genome (*Pseudomonas fluorescens* SBW25
531 NC_012660)⁵³, variants (SNPs and short INDELs) were called using HaplotypeCaller and
532 jointly genotyped for all 10 time points per population using GenotypeGVCFs with GATK
533 (version 3.5) and ploidy set to 30. Thus, for each population, we could detect variants at each
534 locus at a frequency detection limit and resolution of 3.3 % (100 % / 30). Variants were hard-
535 filtered to omit variants with combined read depth < 100 and Phred-scaled quality < 50. We
536 used SnpEff⁵⁴ with the annotation file corresponding to the reference genome for variant effect
537 prediction, i.e. to detect whether the variant has no predicted effects (non-coding variants:

538 intergenic regions and synonymous variants) or results in an amino acid change (all coding,
539 non-synonymous variants). Prior to further analyses, variant counts (max. 30) were converted
540 into frequencies (0–1).

541 We designed a pipeline to remove likely sequence errors from the resulting dataset
542 utilizing previously published pipelines^{25,55} (see also Supplementary Information). To reliably
543 track variant frequency, we excluded variant loci represented by two or more alternate alleles
544 in the same population in GATK variant calling. Since the frequency of a real mutation is
545 expected to be correlated across time points, we excluded variants whose frequency trajectories
546 had a lag-1 autocorrelation < 0.2 . Variants occurring immediately at detectable frequency are
547 more likely to be either ancestral variants or sequence errors compared to variants emerging at
548 later time points. Therefore, initial variants (first two sequenced time points) were required to
549 have a stricter minimum lag-1 autocorrelation of 0.5. Because variants that remain at very low
550 frequencies are unreliable, we required a variant to reach 0.1 frequency in a minimum of two
551 time points. We also excluded variants located within 10 bp from INDELS, which might have
552 an increased likelihood of being alignment errors. Finally, to ensure that the data has sufficient
553 temporal resolution, we removed variants with missing information from over two ($> 2/10$)
554 time points (resulting e.g. from insufficient coverage at the variant locus in a given sample).

555 As well as analysing mutations individually, we assigned them to cohorts, i.e. temporal
556 clusters of mutations, using a previously developed approach²⁵. First, a Euclidian distance
557 matrix was created from frequency vectors of mutations with ≥ 0.3 maximum frequency, since
558 low-frequency mutations cannot be reliably clustered. The distance matrix was hierarchically
559 clustered, and the hierarchies were flattened using a cutoff distance of 0.5 (data resolution did
560 not permit lower cutoff distances), using the `dist`, `hclust` and `cutree` functions in the `stat` package
561 in base R. After all filtration steps, we also extracted nonsynonymous candidate mutations
562 potentially under selection from variant data based on being located on a gene mutated at min.

563 50 % frequency in at least one non-mutator population (to leave out nonselected hitchhikers
564 likely present in the mutational cohorts of mutator populations).

565 In addition to detection of SNPs and short INDELs using the approach outlined above,
566 we performed read-depth-based detection of large genomic deletions and duplications (i.e.
567 copy number variation, CNV) using cnvator 0.3.2⁵⁶ with a bin size of 500 bp. CNVs of interest
568 were extracted based on absence in the first sequenced time point (likely ancestral CNV or
569 sequence error) and detection in at least two consecutive time points (signal of potential
570 selection).

571 To test for differences in the total number of variants and the number of filtered variants
572 between treatments, we use generalized linear models with the family Poisson and the link
573 function log with treatment as factor. We used glms with the family Gamma and the link
574 function inverse to test for the distribution of variants (total and filtered) in different impact
575 classes with the presence and absence of streptomycin and predators and impact class as
576 factors.