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1 # Artificial selection of microbial communities to enhance
2 # degradation of recalcitrant polymers

3

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12

13 ## Author contributions

14 RW and JCO designed the study. RW performed all experiments with guidance from JCO and

15 MIG. RW wrote the first draft of the manuscript and all authors contributed substantially to

16 revisions. The authors declare no conflicts of interest.

17

18 ## Keywords (up to ten)

19 Artificial selection; Microbial community; Polymer degradation; Chitin degradation; Marine

20 systems; Ecological succession

21

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22 **Data accessibility**: Should this manuscript be accepted, we will make all supporting data and
23 figures available through Dryad. All sequences have been deposited in the NCBI Short Read
24 Archive (SRA) database under Bioproject PRJNA499076.
25

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37 ## Abstract

38 Recalcitrant polymers are widely distributed in the environment. This includes natural
39 polymers, such as chitin, but synthetic polymers are becoming increasingly abundant, for
40 which biodegradation is uncertain. Distribution of labour in microbial communities commonly
41 evolves in nature, particularly for arduous processes, suggesting a community may be better
42 at degrading recalcitrant compounds than individual microorganisms. Artificial selection of
43 microbial communities with better degradation potential has seduced scientists for over a
44 decade, but the method has not been systematically optimised nor applied to polymer
45 degradation. Using chitin as a case study, we successfully selected for microbial communities
46 with enhanced chitinase activities but found that continuous optimisation of incubation times
47 between selective generations was of utmost importance. The analysis of the community
48 composition over the entire selection process revealed fundamental aspects in microbial
49 ecology: when incubation times between generations were optimal, the system was
50 dominated by *Gammaproteobacteria*, main bearers of chitinase enzymes and drivers of chitin
51 degradation, before being succeeded by cheating, cross-feeding and grazing organisms.

52 **Importance**

53 Artificial selection is a powerful and atractive technique that can enhance the biodegradation
54 of a recalcitrant polymer and other pollutants by microbial communities. We show, for the
55 first time, that the success of artificially selecting microbial communities requires an
56 optimisation of the incubation times between generations when implementing this method.
57 Hence, communities need to be transferred at the peak of the desired activity in order to
58 avoid community drift and replacement of the efficient biodegrading community by cheaters,
59 cross-feeders and grazers.

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60 ## 1. Introduction

61 Recalcitrant compounds are widely distributed in the environment (1–6). These include natural
62 polymers, such as cellulose, (7) and chitin (1), and, more recently, xenobiotic compounds like plastics
63 (2, 3, 5, 8), pesticides and detergents (9). Whilst processes to degrade natural compounds have had
64 time to evolve and adapt, these processes may still require the participation of a consortia of
65 organisms, each specialised in one of the multiple steps involved in the breakdown of the compound
66 (10, 11). Laborious biodegradation processes are therefore rarely carried out entirely by a single
67 microorganism in nature, and it is now well documented that a distribution of labour is favoured in
68 natural microbial communities (12–16).

69

70 Many xenobiotic compounds have only existed in the last 50-100 years and microbial communities
71 have had little time to evolve efficient biodegradation pathways to catabolise them. Some novel
72 enzymes have, however, been discovered for new xenobiotic compounds, such as the recent
73 discovery of an esterase involved in the degradation of poly(ethylene terephthalate) (PET) (17). This
74 enzyme, termed “PETase”, is thought to have evolved from other esterases i.e. lipases and cutinases.
75 Hence, although this enzyme shares considerable sequence homology with other enzymes capable
76 of PET degradation (17–19), it has developed a higher hydrolytic activity against this polymer than
77 any other tested esterase but, still, there is room for evolutionary improvement (18). The bacterium
78 encoding this enzyme was isolated from a PET-degrading consortia of microorganisms and is capable
79 of metabolising PET to its monomers, terephthalic acid and ethylene glycol (17). Similarly to the
80 generation of toxic phenolic intermediates during lignin degradation (20), terephthalic acid can
81 become toxic at high concentrations (21, 22) which suggests that degradation could be more efficient
82 if carried out by a consortium rather than an individual organism. There are a number of examples

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83 of microbial consortia assembled to degrade recalcitrant xenobiotic compounds e.g. phthalic acid
84 esters, benzene, xylene and toluene (23); polychlorinated biphenyls (24); polystyrene (25); and
85 polyethylene (26) but, due to adverse biotic and abiotic conditions (e.g. temperature, humidity,
86 competition and predation), the natural evolutionary development of novel biodegrading pathways
87 and/or microbial consortia may be hampered in the environment (27).
88
89 Faster evolution can be achieved through artificial selection. A whole microbial community may be
90 used as a unit of selection (artificial ecosystem selection) so that it becomes progressively better at
91 a selective process over successive generations (28–31). The artificial selection of a measurable and
92 desired trait is thought to outperform traditional enrichment experiments as it bypasses community
93 bottlenecks and reduces stochasticity (31). Artificial ecosystem selection has been implemented for
94 developing a microbial community capable of degrading the toxic environmental contaminant 3-
95 chloroaniline (29) as well as to lower carbon dioxide emissions during growth (31) and generating a
96 microbial community adapted to low or high soil pH (28). However, to our knowledge, it has not been
97 previously used for improving polymer degradation and nor have the growth parameters involved
98 (*e.g.* incubation time) been systematically optimised to enhance the selectivity of a desired process.
99
100 In the present study we aimed to optimise the artificial selection process of a marine microbial
101 community for polymer degradation, using chitin as a case study. Chitin is one of the most abundant
102 polymers on Earth (*i.e.* the most abundant polymer in marine ecosystems) constituting a key
103 component in oceanic carbon and nitrogen cycles (1). Many microorganisms are already known to
104 degrade chitin, and the enzymes and pathways used to do so are well characterised (10). We found
105 that a microbial community could be artificially evolved to achieve higher chitinase activities, but
106 there were certain methodological caveats to this selection process. We found that the incubation

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107 time between generations needed to be continuously optimised in order to avoid community drift
108 and decay. Microbial community composition was evaluated and we confirmed that, if generation
109 times are not continuously optimised, efficient biodegrading communities are rapidly taken over by
110 cross feeders and predators with a subsequent loss of degrading activity.

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111 # Results

112 **First artificial selection experiment; process optimisation**

113 Our first artificial selection experiment highlighted the need to sub-culture each generation when the
114 desired trait/chitinase activity was at its peak and not at a pre-defined incubation time, as done
115 previously (28, 29, 31). Initially, we set a standardised nine-day incubation time for each generation
116 because this was the time it took for chitinase activity to peak in a preliminary enrichment
117 experiment (data not shown). After 14 generations we did not observe a strong increase in chitinase
118 activity (Fig. 2A, and Supplementary Fig. S1) and, intriguingly, in nine out of the 14 generations we
119 observed a lower activity in the positive selection than in the randomly selected control (Fig. 2A),
120 suggesting that a random selection of microcosms is more effective in enhancing chitinase activity
121 than actively selecting for the best communities. To further investigate the reasons behind this low
122 efficiency, we took regular enzymatic activity measurements within generation 15 (Fig. 2B). As
123 suspected, the chitinase activity was peaking much earlier within the generation, *i.e.* at day four, and
124 by the end of the nine days the chitinase activity had dropped below the activities registered for the
125 random selection experiment (Fig. 2B). Attending to this result, at generation 16 we set up an
126 additional experiment, run in parallel, where the incubation time per generation was shortened to
127 four days. Shortening the incubation time led to a selection of higher chitinase activities during
128 generations 16 and 17, but the progressive increase in activity stalled by generations 18 and 19 (Fig.
129 2A). Chitinase activity was again measured daily within the final generation 20 and we found that the
130 enzymatic activity was almost nine times higher on day two than day four (Fig. 2C), indicating that
131 the optimal incubation time had again been reduced. While the nine-day incubation experiment gave
132 an overall negative trend, shortening the incubation times to the chitinase maxima drastically
133 increased the benefits of artificial community selection (Fig. 2A).

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134

135 **Microbial community succession**

136 We carried out MiSeq amplicon sequencing of the 16S and 18S rRNA genes to characterise the
137 microbial community succession that occurred within the first selection experiment and, by this way,
138 gain insight into the strong variability in chitinase activity observed over time. We sequenced the
139 communities that were used as the inoculum of each of the 20 generations, both nine and four-day
140 long experiments, as well as the community obtained from the daily monitoring of generation 20.
141 This data was processed using both Mothur (32) and DADA2 workflows (33, 34), obtaining similar
142 results (Supplementary Figs. S2 and S3). DADA2 results are presented here as this workflow retains
143 greater sequence information, better identifies sequencing errors and gives higher taxonomic
144 resolution (35). Unique taxa are therefore called amplicon sequence variants (ASVs) rather than
145 operational taxonomic units (OTUs).

146

147 *Community succession over the four-day incubation period within generation 20.*

148 The daily microbial community analysis over the four days at generation 20 showed a progressive
149 increase in prokaryotic diversity (from 0.83 to 0.93, according to Simpsons index of diversity) whereas
150 a strong decrease in diversity was observed amongst the eukaryotic community (from 0.93 to 0.38;
151 Fig. 3A). SIMPER analyses were carried out to identify those 16S and 18S rRNA gene ASVs that were
152 contributing most to the differences over the four successive days observed in Fig. 3B. The top five
153 ASVs in these analyses were responsible for 50% and 60% of variation for the 16S and 18S rRNA
154 genes, respectively (Fig. 3C).

155

156 For the 16S rRNA gene, the most important ASVs were: ASV3 (*Thalassotalea*, contributing to 16% of
157 the community variation between the four days, p=0.025), ASV4 (Cellvibrionaceae, 15% variation,

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158 *p*=0.033), ASV5 (*Crocinitomix*, 8% variation, *p*=0.033), ASV7 (*Terasakiella*, 6% variation, *p*=0.094) and
159 ASV2 (*Spirochaeta*, 5% variation, *p*=0.022) (Fig. 3C). ASVs 3 and 4 (both *Gammaproteobacteria*)
160 represented over 50% of the prokaryotic community abundance on day 2, when chitinase activity
161 was highest, and their abundances followed a similar pattern to the chitinase activity over the four
162 days (Fig. 2C), suggesting that these ASVs may be the main drivers of chitin hydrolysis. On the other
163 hand, ASVs 7 and 2 both showed a progressive increase over time (*i.e.* from a combined relative
164 abundance of 5% on day 1 to 23% on day 4; Fig. 3C), suggesting that these ASVs could be cross-
165 feeding organisms that benefit from the primary degradation of chitin. Interestingly, the overall 16S
166 rRNA gene analysis also showed a strong succession over time at higher taxonomic levels (Fig. 4).
167 While *Gammaproteobacteria* pioneered and dominated the initial colonisation and growth,
168 presumably, via the degradation of chitin (*i.e.* with 73% relative abundance during the first two days),
169 all other taxonomic groups became more abundant towards the end of the incubation period (*e.g.*
170 *Clostridia*, *Bacteroidia* and *Alphaproteobacteria* increased from an initial relative abundance of 0.1,
171 2.8 and 12% on day one to 13.5, 22 and 21% on day four, respectively; Fig. 4). Microbial isolates
172 confirmed *Gammaproteobacteria* as the main contributors of chitin-biodegradation (as discussed
173 below).
174
175 The SIMPER analysis of the 18S rRNA gene highlighted ASV2 (*Cafeteria* sp., contributing to 34% of the
176 community variation between the four days, p=0.016), ASV4 (*Paraphysomonas*, 10% variation,
177 p=0.023), ASV1 (*Cafeteria* sp., 6% variation, p=0.392), ASV6 (*Apsidica*, 5% variation, p=0.040) and
178 ASV3 (Incertae Sedis, 5% variation, p=0.059) as the five main ASVs contributing to 60% of the
179 community variation over the four days (Fig 3C). ASV2, which was 96% similar to the bactivorous
180 marine flagellate *Cafeteria* sp., was by far the most striking Eukaryotic organism, showing an increase
181 in relative abundance from 2% on day 1 up to over 76% on day 4 (Fig. 3B and 3C). As observed in

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182 prokaryotes, Eukaryotic phylogenetic groups also showed a large variation between the beginning
183 and the end of the incubation period, mainly due to the increase of *Bicosoecophyceae* over time (*i.e.*
184 from 2.6 to 89% relative abundance driven by both ASV1 and ASV2; Supplementary Fig. S4).
185
186 *Community succession over the entire artificial selection experiment.*
187 We analysed the 16S and 18S rRNA gene community composition (Supplementary Fig. S5) at the end
188 of each generation in order to determine the effect that positive or random selection of communities
189 had across the 20 generations, both for the nine-day incubation experiment (*i.e.* generations 0 to 20)
190 and shortened four-day incubation experiment (*i.e.* generations 16 to 20). Most interestingly, the
191 overall community variability across all generations (16S and 18S rRNA gene nMDS analysis; Fig. 5A)
192 showed that only the positive selection of the shortened four-day incubations differentiated the
193 community from the random selection, which was confirmed by a PerMANOVA test using Bray-Curtis
194 distance (16S rRNA gene p=0.001; 18S rRNA gene p=0.002; Supplementary Table S2), while the nine-
195 day selection mostly clustered with the random control communities. This is a clear explanation as
196 to why the nine-day incubation time was not allowing a progressive selection of a community with
197 better chitinase activities than those obtained randomly and, only when the time was shortened, did
198 we observe an effect of the positive selection over the random selection.
199
200 SIMPER analyses were carried out to determine the ASVs that most strongly contributed to the
201 differences between groups (*i.e.* positive *versus* random selections and nine-day *versus* four-day
202 incubation times; Fig. 5B). For the 16S rRNA gene, the top five ASVs identified by the SIMPER analysis
203 contributed to 35% of the community variation, while for the 18S rRNA gene, they accounted for 61%
204 (Fig. 5B). The 16S rRNA gene ASVs 5, 7 and 11 (*Crocinitomix*, *Terasakiella* and *Carboxylicivirga flava*,
205 respectively) presented a much higher abundance in the four-day positive selection than in any other

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206 selection (13%, 11% and 8%, respectively), suggesting that these species were the major contributors
207 to the differentiation of these communities, as seen in Fig. 5A. As observed above for the four-day
208 incubation analysis, *Cafeteria* sp. (18S rRNA gene ASV1 and ASV2, both 96% similar) was again the
209 most conspicuous Eukaryotic organism. ASV2 was more abundant in the positive four-day selection
210 (32% of the relative abundance), while ASV1 was highest in the three other selections (70% and 82%
211 in the positive and random nine-day selection, respectively, and 16% in the random four-day
212 selection; Fig. 5B).
213
214 *Chitinase gene copies in artificially-assembled metagenomes*
215 Artificially-assembled metagenomes, generated by PICRUSt (36) from the 16S rRNA gene amplicon
216 sequences, were used to search for enzymes involved in chitin degradation: KEGG orthologs K01183
217 for chitinase, K01207 and K12373 for chitobiosidase, K01452 for chitin deacetylase, and K00884,
218 K01443, K18676 and K02564 for the conversion of GlcNAc to Fructose-6 phosphate (Supplementary
219 Fig. S6) (37–39). As expected from the measured chitinase activities, the shortened four-day
220 incubation experiment showed over 30 times more chitinase (K01183) gene copies than the nine-day
221 incubation experiment (*i.e.* an average of 0.66 copies per bacterium were observed in the four-day
222 incubation experiment while only 0.025 copies per bacterium were observed over the same
223 generations in the nine-day experiment). Also, from the daily analysis of generation 20, the chitinase
224 activity was positively correlated with the normalised chitinase gene copy number (r^2=0.57), with a
225 peak in chitinase activity *and* chitinase gene copies on day 2 (*i.e.* over one chitinase gene copy per
226 bacterium). The most striking result from this analysis was the strong bias of taxonomic groups that
227 contributed to the chitinase and chitin deacetylase genes; chitinase genes were mainly detected in
228 *Gammaproteobacteria* and some *Bacteroidia*, whereas the chitin deacetylase genes were almost
229 exclusively present in *Alphaproteobacteria*. It is worth highlighting that the chitosanase gene

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230 (K01233), the enzyme required to hydrolyse the product from chitin deacetylation, chitosan, was not
231 detected in any of the artificial metagenomes. Chitobiosidases (K01207 and K12373) and enzymes
232 involved in the conversion of GlcNAc to Fructose-6 Phosphate (K00884, K01443, K18676 and K02564)
233 were more widespread. Nevertheless, this data needs to be taken with caution as these were not
234 real metagenomes.
235
236 *Isolation and identification of chitin degraders*
237 Bacterial isolates were obtained from the end of the artificial selection experiments to confirm the
238 ability of the identified groups to degrade chitin. From the 50 isolates obtained, 20 were unique
239 according to their 16S rRNA gene sequences. From these, 18 showed at least 98% similarity with one
240 or more of the MiSeq ASVs (Supplementary Table S3) although, unfortunately, none belonged to the
241 most abundant ASVs detected during the community analysis. The ability for chitin and GlcNAc
242 degradation by each one of the isolates was assessed. We found that 16 of these isolates could grow
243 using GlcNAc as the sole carbon source, but only 11 of these strains could grow on chitin (Fig. 4). The
244 four remaining bacteria from the 20 isolated could not grow using chitin or GlcNAc. Most
245 interestingly, all isolates from the class *Gammaproteobacteria* (*n*=7) were capable of chitin
246 degradation whereas only a smaller subset of isolates had this phenotype in other abundant
247 taxonomic groups, such as *Bacteroidia* (1 out of 3) or *Alphaproteobacteria* (1 out of 8; Fig. 4).
248
249 **Second artificial selection experiment; implementing an improved selection process**
250 A second selection experiment showed an extremely rapid boot in chitinase activity *i.e.* reaching
251 almost 90 μM day^{-1} in only 7 generations (Figs. 6, and S7), when the maximum activity achieved in
252 the first experiment was 0.9 μM day^{-1} (Fig. 2C), demonstrating that implementing an optimised
253 incubation time between generations largely enhances the selection of a desired trait. Chitinase

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254 activity was measured daily until a peak in chitinase activity was observed. The communities with

255 highest chitinase activity on this day were used to start the next generation.

256

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257 # Discussion

258 Artificial selection of microbial communities is, in principle, a powerful and atractive technique which
259 has surprisingly been used in only a limited number of studies to date (28, 29, 31), possibly due to
260 the lack of success as a consequence of poor process optimisation. Here, using chitin degradation as
261 a case study and a detailed analysis of the community succession, we show that artificial selection of
262 microbial communities can be largely improved by controlling the incubation times between
263 generations. The rapid succession of microbial community structure means generations need to be
264 transferred at the peak of the selected phenotypic activity (*e.g.* chitinase activity) or these get rapidly
265 replaced by less efficient communities of cross-feeding microorganisms (*i.e.* bacteria and grazers).
266 Previous studies that have artificially selected microbial communities for a particular phenotype did
267 not report optimisation of the incubation time between generations (28, 29, 31) which, in our hands,
268 would have resulted in a negative selection (Fig. 2). In agreement with our results, Penn and Harvey
269 (2004) (40) suggested that the observed phenotype in artificial ecosystem selection experiments
270 could be significantly affected by community structure.

271

272 An understanding of microbial ecology helps explain the importance of the timing during generation
273 transfer. Datta et al. (2016) (42) observed three distinct stages of community structure during the
274 colonisation of chitin particles: (a) attachment; (b) selection, and; (c) succession. Each phase was
275 characterised by having relatively higher abundances of organisms that were: (a) good at attaching
276 to chitin particles; (b) good at degrading chitin particles, and; (c) not able to degrade chitin, but able
277 to benefit from others that could, *i.e.* "cheaters" and cross-feeders (43, 44). During our first
278 experiment, as communities become better and faster at degrading chitin, we were measuring the
279 chitinase actvity when the communities were in the succession rather than in the selection stage and,

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280 therefore, the active chitinolytic community had decayed and was dominated by cheaters and cross-
281 feeders (Figs. 3 and 4). Hence, it was only when selecting at phenotypic time optima when chitinase
282 activity improved and the overall community differentiated from the random control communities
283 (Fig. 5 and 6). It is also interesting to note the selection of the grazer *Cafeteria* sp. (90% of the
284 Eukaryotic community), a genus of bactivorous marine flagellates that are commonly associated with
285 marine detritus (45). The predator-prey dynamics postulated by Lotka–Volterra's equations would
286 also support the need to shorten generation times to favour the prey's growth *i.e.* chitinolytic
287 bacteria (46, 47).
288
289 Interestingly, a strong successional pattern was observed at a higher taxonomic level. While
290 *Gammaproteobacteria* dominated during the initial stages when chitinase activity was at its peak
291 (accounting for over 70% of the prokariotic community), other groups increased in abundance during
292 the later stages (*i.e. Alphaproteobacteria*, *Bacteroidia* and *Clostridia*), similarly to the pattern
293 previously observed by Datta et al. (2016) (42).
294
295 The fact *Gammaproteobacteria* are major contributors to chitin degradation is not new (48–53). All
296 *Gammaproteobacteria* isolates obtained from the end of the experiments were able to grow using
297 chitin as the only source of carbon and energy (Fig. 4) confirming that this class is likely responsible
298 for most of the chitinase activity observed. On the other hand, *Alphaproteobacteria*, the numerically
299 dominant class of heterotrophic bacteria in surface oceans (54, 55), follow a cross-feeding and/or
300 cheating life-strategy as five out of eight *Alphaproteobacterial* isolates could only use N-acetyl-D-
301 glucosamine (GlcNAc) and only one could use chitin. This was confirmed by the PICRUSt metagenome
302 analysis (Fig. S6), where almost all chitinase enzymes copies were encoded by *Gammaproteobacteria*
303 (*i.e.* 90%; encoding almost one gene copy per bacterium) and, to a lesser extent, by some *Bacteroidia*.

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304 Chitin is made up of molecules of GlcNAc linked by (1,4)-β-glycosidic bonds, and it has previously
305 been found that initial degradation of chitin takes place predominantly by: i) chitinases which
306 depolymerise the (1,4)-β-glycosidic bonds either at the ends or in the middle of chains, or ii)
307 chitobiosidase enzymes which also hydrolyse (1,4)-β-glycosidic bonds but only at the ends of chitin
308 chains. Genes for the intracellular enzymes involved in GlcNAc utilisation (*i.e.* transformation of
309 GlcNAc to Fructose-6-phosphate) were much more widespread amongst different taxonomic groups,
310 highlighting the broader distribution of cross-feeding or cheating organisms which can benefit from
311 the extracellular depolymerisation of chitin which generates freely available GlcNAc to the
312 community. Alternative degradation of chitin may also occur by deacetylation and deamination of
313 the GlcNAc amino sugar, transforming chitin into chitosan and cellulose, respectively, after which
314 they can be depolymerised by a range of other enzymes (*e.g.* chitosanases or cellulases) (10, 56, 57).
315 While *Alphaproteobacteria* did not contribute to chitinase enzymes, it *did* potentially encode for
316 most of the chitin deacetylases in the system, although no chitosanases were detected.
317
318 Chitinolytic organisms have previously been found to make up between 0.1 and almost 6% of
319 prokaryotic organisms in aquatic ecosystems (43, 58), while over a third of the organisms in these
320 habitats can utilise only the products of chitin hydrolysis (*i.e.* GlcNAc) (43, 59–61). With
321 *Gammaproteobacteria* being primarily responsible for the degradation of chitin here, the success of
322 the artificial selection for an enhanced chitinolytic community was possibly achieved by the selective
323 enrichment of this group between the beginning (5% of the prokaryotic community, within the
324 expected range of *Gammaproteobacteria* found within natural environments) (43, 58) and end of the
325 experiment (75% of the community).
326

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327 Here we have proven the validity of artificially selecting a natural microbial community to better
328 degrade a recalcitrant polymer, but have highlighted the caveats for achieving this goal, which
329 require a better understanding of the ecology of the system. We found that optimisation of
330 incubation times is essential in order to successfully implement this process, as optimal communities
331 enter rapid decay due to their replacement by cheaters and cross-feeders, as well as the increase of
332 potential predators such as grazers and, although not tested here, viruses. Hence, future artificial
333 selection experiments should adjust generation incubation times to activity maxima to successfully
334 evolve enhanced community phenotypes.
335

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336 # Materials and methods

337 **Microbial inoculum**

338 The microbial community used as an inoculum was obtained from bulk marine debris collected during
339 boat tows from both Plymouth Sound (Devon, UK; June 2016) and Portaferry (Northern Ireland, UK;
340 August 2016).

341

342 **Chitinase activity measurements**

343 Chitinase activity was measured as the liberation of the fluorogescent molecule 4-methylumbelliferyl
344 (MUF) from three chitinase substrates (MUF-N-acetyl-β-D-glucosaminide, MUF-β-D-N,N'-
345 diacetylchitobioside and MUF-β-D-N,N',N''-triacetylchitotrioside; Sigma Aldrich, UK), following the
346 previously described method (49, 62, 63) (Supplementary information). Standards curves were
347 obtained using chitinase from *Streptomyces griseus* (Sigma Aldrich, UK) dissolved in sterile phosphate
348 buffered saline solution (pH 7.4; 0.137 M) with a highest concentration of 0.1 U mL^{-1} (activity
349 equivalent to 144 µM day^{-1}). Samples were diluted prior to measurement if they were expected to
350 be above this range.

351

352 **Artificial selection**

353 The process for artificial selection is depicted in Figure 1. Briefly, 30 individual microcosms per
354 treatment and generation were incubated in the dark under the conditions described below. At the
355 end of each generation the three microcosms with the highest chitinase activities (or three random
356 microcosms in the case of the control) were pooled and used as the inoculum for the next generation
357 of microcosms (n=30). This was repeated across multiple generations. Two artificial selection

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358 experiments were performed, the first to optimise the process, and the second to implement optimal
359 conditions and achieve a high-performing chitinolytic microbial community:
360
361 *First artificial selection experiment.*
362 Incubations were carried out at 23˚C in 22 mL glass vials (Sigma Aldrich), each containing 20 mL of
363 autoclaved seawater (collected from outside Plymouth Sound, Devon, UK; June 2016) supplemented
364 with NaH_2PO_4, F/2 trace metals (64) (Supplementary information) and 100 mg of chitin powder (from
365 shrimp shells; Sigma Aldrich) as the sole source of carbon and nitrogen. Generation 0 was started
366 with 200 µL of microbial inoculum. The efficiency of the selection process was assessed by comparing
367 a 'positive selection' (where the three communities with highest activity were pooled and 200 µL was
368 used to inoculate each one of the 30 microcosms of the next generation) against a 'random selection'
369 (where three communities were chosen at random, using a random number generator within the
370 Python module Random, to inoculate the following generation) to give a control against
371 uncontrollable environmental variation (65). Each treatment was repeated across 20 generations
372 with incubation times of nine days. In parallel, treatments where incubation times were shortened
373 to four days were setup after generation 15. Samples were taken from each community and stored
374 in 20% glycerol at -80˚C for further microbial isolation, and pellets from 1.5 ml of culture were
375 collected by centrifugation (14,000 *x g* for 5 mins) and stored at -20˚C for final DNA extraction and
376 community analysis.
377
378 *Second artificial selection experiment.*
379 A second selection experiment was setup implementing optimal generation times. Microcosms were
380 incubated in 2 mL 96-well plates (ABgene ™, ThermoFisher Scientific) covered by Corning®
381 Breathable Sealing Tapes to stop evaporation and contamination while allowing gas exchange. Each

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382 well contained 1.9 mL of a custom mineral media containing $MgSO_4$, $CaCl_2$, KH_2PO_4, K_2HPO_4, 0.52 M
383 NaCl and artificial seawater trace metals (Supplementary information), supplemented with 10 mg of
384 chitin powder. The microbial inoculum was 100 µL (*i.e.* initial inoculum and transfer between
385 generations). Chitinase activity was measured daily. Transfer between generations was carried out
386 just after the peak of chitinase activity had occurred, calculated as the average chitinase activity
387 across the 30 microcosms of the positive selection treatment. Plates were incubated in the dark at
388 30˚C with constant shaking (150 rpm). Eight days was the maximum incubation time allowed to reach
389 maximum chitinase activity due to volume constraints.
390
391 **DNA extraction and amplicon sequencing**
392 DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) protocol, with modifications as follows
393 (adapted from 66): 300 µL 1 x TAE buffer was used to resuspend cell pellets and these were added to
394 ~0.4 g of sterile 0.1 mm Biospec zirconia silica beads in 2 mL screw cap microtubes (VWR
395 international). Bead beating was carried out for 2 x 45 s and 1 x 30 s at 30 Hz using a Qiagen Tissue
396 Lyser. Cell lysates were then processed in accordance with the manufacturer's instructions, with an
397 extra centrifugation step to ensure all liquid was removed (1 min, 13,000 *x g*) directly before elution
398 of samples. A Qubit® HS DNA kit (Life Technologies Corporation) was used for DNA quantification
399 after which they were diluted to equalise the concentrations across samples. A Q5® Hot Start High-
400 Fidelity 2X Master Mix (New England Biolabs® inc.) was used to amplify the 16S rRNA gene v4-5
401 regions using primers 515F-Y and 926R (67), and the 18S rRNA gene v8-9 regions using primers V8F
402 and 1510R (68) (Supplementary information). PCR products were purified using Ampliclean magnetic
403 beads (Nimagen, The Netherlands). Index PCR was carried out using Illumina Nextera Index Kit v2
404 adapters. Samples were normalised using a SequelPrep™ Normalisation Plate Kit (ThermoFisher
405 Scientific). Samples were pooled and 2 x 300 bp paired-end sequencing was carried out using the

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406 MiSeq system with v3 reagent kit. Negative and chitin only DNA extraction controls and library
407 preparation negative controls were processed and sequenced alongside samples.
408
409 **Microbial community structure determination**
410 Two different workflows were used to analyse the sequencing data: DADA2 (33, 34) and Mothur (32).
411 DADA2 delivers better taxonomic resolution than other methods (*e.g.* Mothur) as it retains unique
412 sequences and calculates sequencing error rates rather than clustering to 97% similarity (35). The
413 resultant taxonomic units are referred to as amplicon sequence variants (ASVs) rather than
414 operational taxonomic units (OTUs from Mothur). For the DADA2 analysis, sequencing data were
415 processed following the DADA2 (version 1.8.0) pipeline (33). Briefly, the data were filtered, *i.e.*
416 adapter, barcode and primer clipped, and the ends of sequences with high numbers of errors were
417 trimmed. The amplicons were denoised based on a model of the sequencing errors and paired end
418 sequences were merged. Only sequences between 368 - 379 for the 16S rRNA gene and 300 - 340 for
419 the 18S rRNA gene were kept and chimeras were removed. The resulting ASVs were classified using
420 the SILVA reference database (v132) (69). For the Mothur analysis (32), sequencing data were filtered
421 *i.e.* adapter, barcode and primer clipped, sequence length permitted was 450 bp for the 16S rRNA
422 gene and 400 bp for the 18S rRNA gene, maximum number of ambiguous bases per sequence = 4,
423 maximum number of homopolymers per sequence = 8. Taxonomy assignment was performed using
424 the SILVA reference database (Wang classification, v128) (69) and operational taxonomic units
425 (OTUs) set at 97% similarity. For both processing workflows, chloroplasts, mitochondria and
426 Mammalia were removed from the 16S rRNA gene and 18S rRNA gene datasets, eukaryotes were
427 removed from the 16S rRNA gene dataset, and bacteria and archaea from the 18S rRNA gene dataset.
428 The average number of reads per sample was approximately 12,500 for the 16S rRNA gene and
429 20,000 (Mothur) or 34,000 (DADA2) for the 18S rRNA gene. Samples with less than 1,000 total reads

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430 were excluded from downstream analyses. Although most analyses were carried out using relative
431 abundance, each sample was subsampled at random to normalise the number of reads per sample,
432 and the resulting average coverage was 92% (Mothur) or 94% (DADA2) for the 16S rRNA gene and
433 99% (Mothur and DADA2) for the 18S rRNA gene.
434
435 **Microbial isolation and characterisation**
436 Microbes were isolated from the final generation of positive selection experiments by plating serial
437 dilutions on Marine Broth 2216 (BD Difco™) and mineral medium plates (*i.e.* custom medium;
438 Supplementary information) supplemented with 0.1% N-acetyl-D-glucosamine (GlcNAc) and 1.5%
439 agar. Colonies were re-streaked on fresh agar plates until pure isolates were obtained. The
440 identification of isolates was carried out by sequencing the partial 16S rRNA gene (GATC BioTech,
441 Germany) using primers 27F and 1492R (70) (Supplementary information).
442
443 Isolates were grown in custom mineral medium supplemented with either 0.1% chitin or 0.1% GlcNAc
444 (w/v), as sources of carbon and nitrogen, to test for chitinase activity and chitin assimilation,
445 respectively. Growth was monitored over 14 days by measuring: i) chitinase activity (as described
446 above), ii) optical density at 600 nm, and iii) protein content (following manufacturer's instructions;
447 QuantiPro™ BCA Assay Kit, Sigma Aldrich, UK). Isolates were also tested on custom mineral medium
448 agar plates made with the addition of 0.1% chitin and 0.8% agarose. Plates were incubated at 30˚C
449 for 21 days to allow the formation of halos indicative of chitinase activity.
450
451 **Statistical analyses**
452 All analyses of chitinase activity and most MiSeq data analyses were carried out using custom Python
453 scripts (Python versions 2.7.10 and 3.6.6) using the modules: colorsys, csv, heapq, matplotlib, numpy,

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454 os, pandas, random, scipy, scikit-bio, sklearn (71), and statsmodels. SIMPER analyses and plotting of
455 phylogenetic trees were performed in R (R version 3.3.3) (72) using the following packages: ape (73),
456 dplyr, ggplot2, gplots, ggtree (74), lme4, phangorn (75), plotly, tidyr, vegan (76), phyloseq (77). The
457 top 5 ASVs identified in each SIMPER analyses were classified to their closest relative using a BLAST
458 search of the GenBank database with a representative sequence. Hypothetical community functions
459 were obtained using PICRUSt in QIIME1 (36, 78). Sequences used for phylogenetic trees were aligned
460 using the SILVA Incremental Alignment (www.arb-silva.de) (79) and mid-point rooted maximum
461 likelihood trees were constructed using QIIME1 (78). All scripts can be found at https://github.com/R-
462 Wright-1/ChitinSelection.git. All sequences have been deposited in the NCBI Short Read Archive
463 (SRA) database under Bioproject PRJNA499076.

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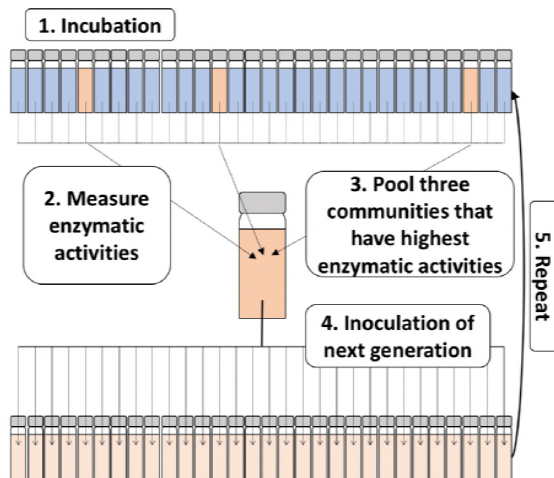


Figure 1. Method used for artificial selection of microbial communities. Briefly, 30 microcosms are inoculated with a natural community found in seawater (1). At the end of the incubation period, the enzymatic activity for a desired trait (*e.g.* chitinase activity) is measured for each microcosm (2). The three microcosms with the highest enzymatic activities are selected and pooled (3), and used to inoculate the next generation (4). This process is repeated over *n* generations (5).

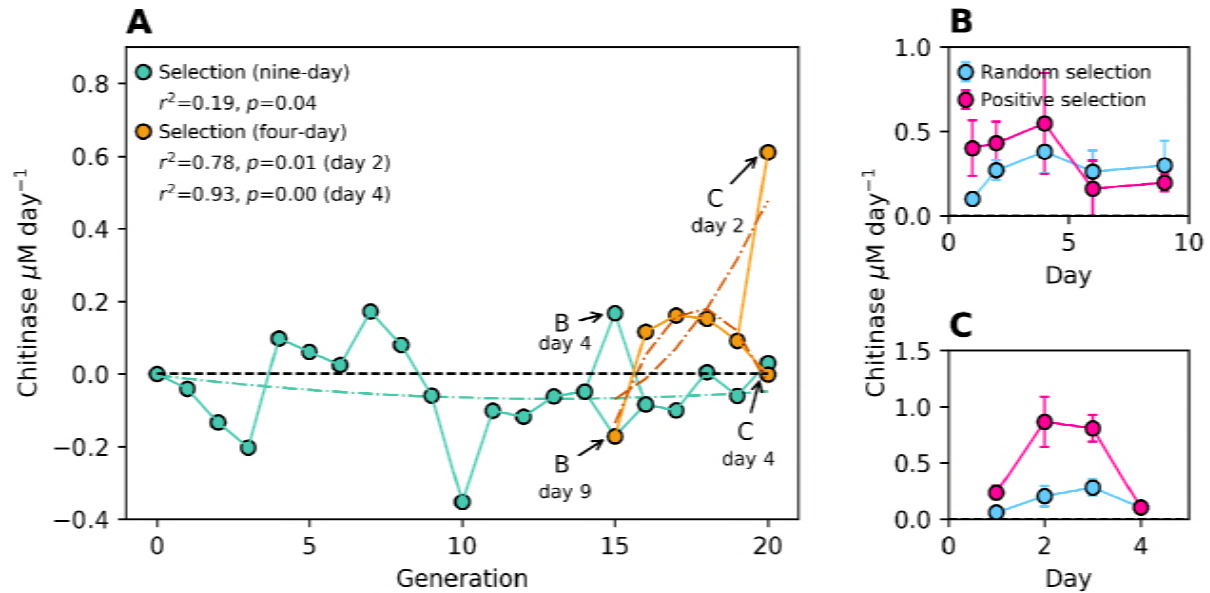
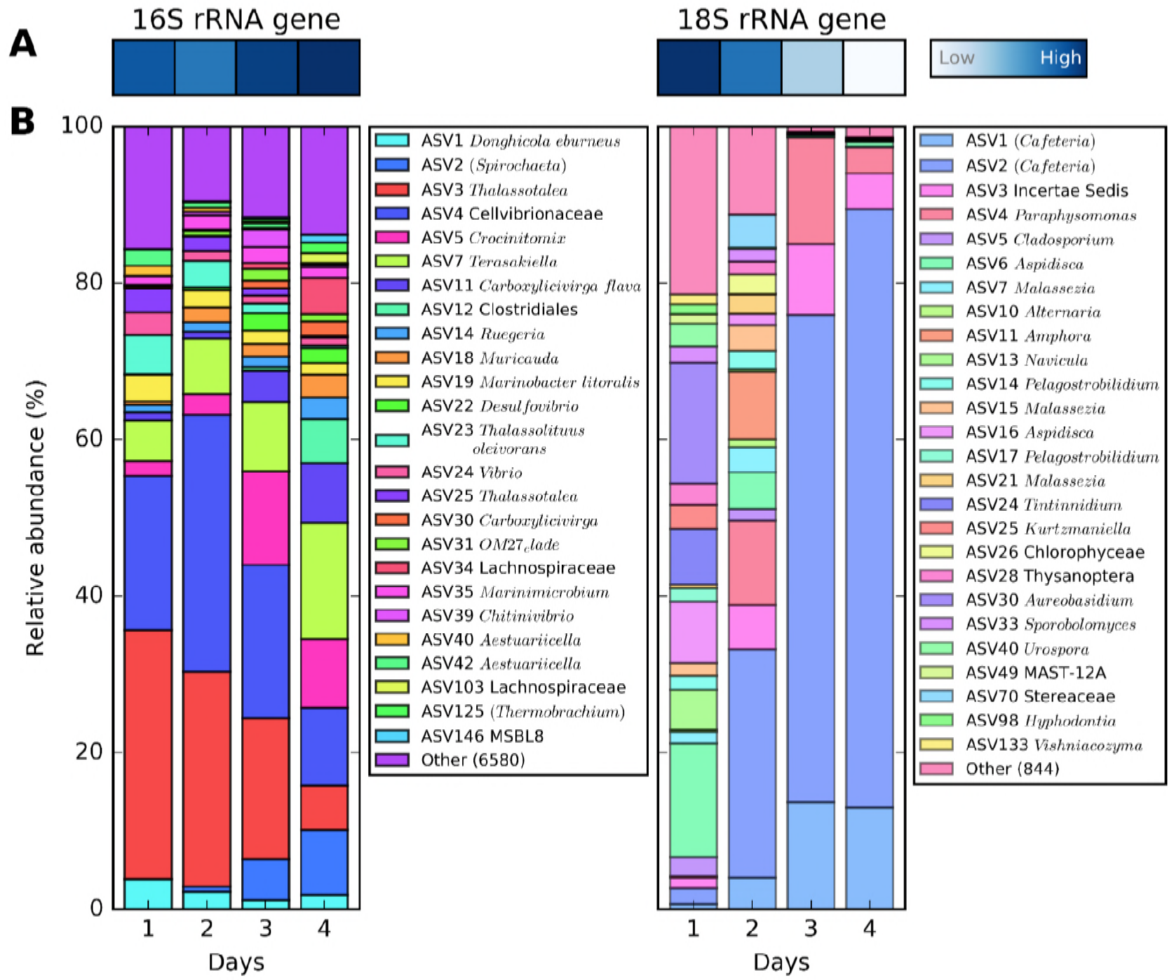


Figure 2. Chitinase activity in artificial selection experiment 1. (**A**) Enzymatic activity measured over 20 generations. Each point represents the mean of the positive selection communities (n=30) to which the mean of the randomly selected controls (n=30) was subtracted. The black dotted line (zero) represents where chitinase activity of the positive selection is equal to that of the random selection. Coloured dotted lines show the general trend for the respective incubation times generated by a linear regression model within Pythons' statsmodels package (green and orange for nine- and four-day incubations, respectively). The r^2 and p-values for Pearson's correlation coefficients between generation number and normalised chitinase activity are shown. (**B**) Chitinase activity measured within generation 15 of the 9-day incubation. (**C**) Chitinase activity measured within generation 20 of the 4-day incubation. In panels B and C, each point represents absolute chitinase activity measured in the positive (red) and random selection (blue).


A
16S rRNA gene
18S rRNA gene
Low
High
B
Relative abundance (%)
ASV1 Donghicola eburneus
ASV2 (Spirochaeta)
ASV3 Thalassotalea
ASV4 Cellvibrionaceae
ASV5 Crocinitomix
ASV7 Terasakiella
ASV11 Carboxylicivirga flava
ASV12 Clostridiales
ASV14 Ruegeria
ASV18 Muricauda
ASV19 Marinobacter litoralis
ASV22 Desulfovibrio
ASV23 Thalassolituus oleivorans
ASV24 Vibrio
ASV25 Thalassotalea
ASV30 Carboxylicivirga
ASV31 OM27_clade
ASV34 Lachnospiraceae
ASV35 Marinimicrobium
ASV39 Chitinivibrio
ASV40 Aestuariicella
ASV42 Aestuariicella
ASV103 Lachnospiraceae
ASV125 (Thermobrachium)
ASV146 MSBL8
Other (6580)
ASV1 (Cafeteria)
ASV2 (Cafeteria)
ASV3 Incertae Sedis
ASV4 Paraphysomonas
ASV5 Cladosporium
ASV6 Aspidisca
ASV7 Malassezia
ASV10 Alternaria
ASV11 Amphora
ASV13 Navicula
ASV14 Pelagostrobilidium
ASV15 Malassezia
ASV16 Aspidisca
ASV17 Pelagostrobilidium
ASV21 Malassezia
ASV24 Tintinnidium
ASV25 Kurtzmaniella
ASV26 Chlorophyceae
ASV28 Thysanoptera
ASV30 Aureobasidium
ASV33 Sporobolomyces
ASV40 Urospora
ASV49 MAST-12A
ASV70 Stereaceae
ASV98 Hyphodontia
ASV133 Vishniacozyma
Other (844)
Days

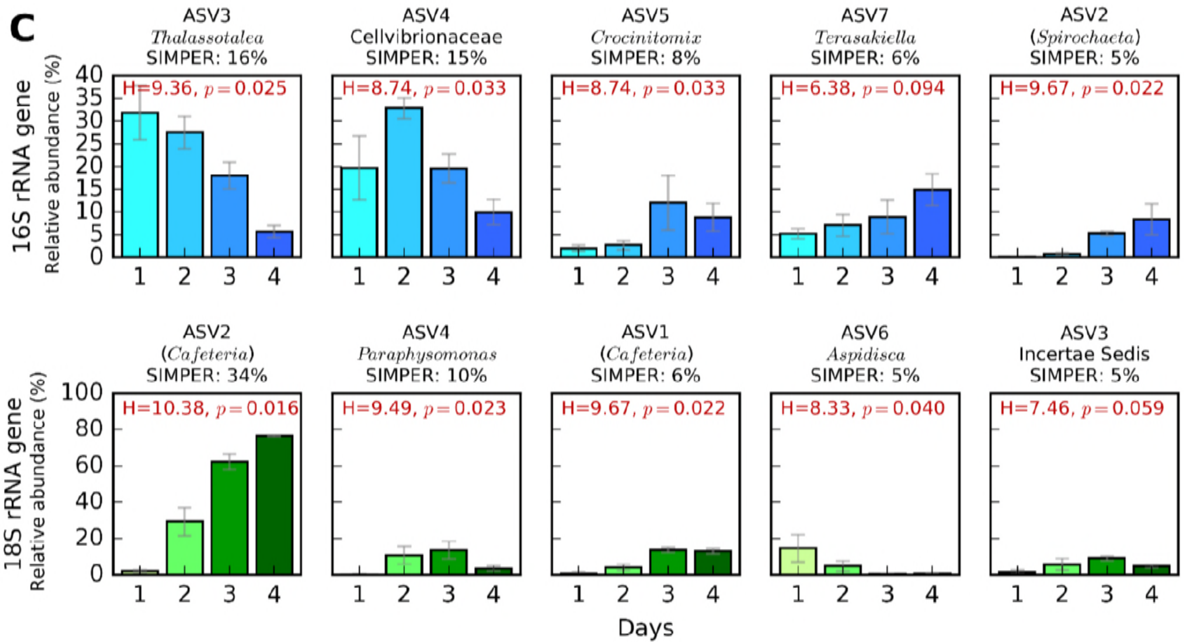

C
16S rRNA gene
Relative abundance (%)
ASV3 Thalassotalea SIMPER: 16%
H=9.36, p=0.025
ASV4 Cellvibrionaceae SIMPER: 15%
H=8.74, p=0.033
ASV5 Crocinitomix SIMPER: 8%
H=8.74, p=0.033
ASV7 Terasakiella SIMPER: 6%
H=6.38, p=0.094
ASV2 (Spirochaeta) SIMPER: 5%
H=9.67, p=0.022
18S rRNA gene
Relative abundance (%)
ASV2 (Cafeteria) SIMPER: 34%
H=10.38, p=0.016
ASV4 Paraphysomonas SIMPER: 10%
H=9.49, p=0.023
ASV1 (Cafeteria) SIMPER: 6%
H=9.67, p=0.022
ASV6 Aspidisca SIMPER: 5%
H=8.33, p=0.040
ASV3 Incertae Sedis SIMPER: 5%
H=7.46, p=0.059
Days

Figure 3. Daily microbial community analysis over the four day incubation period within generation 20. The analysis was performed on the three communities that showed highest chitinase activity by the end of the four days and which would have been used to inoculate the next generation. (**A**) Simpsons index of diversity of the 16S (left) and 18S rRNA gene (right) amplicon analysis. Scale ranges between 0.38 (low) and 0.93 (high). (**B**) Community relative abundance over the 4 day incubation period. Only ASVs with abundance above 1% in at least one time point are shown. The abundance for each ASV is a mean value from the three communities. ASVs were classified to genus level by SILVA. Names in brackets were not identifiable with the standard analysis pipeline and were identified through a BLAST search of the NCBI database. (**C**) Five 16S and 18S rRNA gene ASVs that contributed the most to the community variations over time according to a SIMPER analysis. The percentage of variation to which each ASV contributes is indicated. Error bars represent the standard deviations of three communities used to inoculate the next generation.

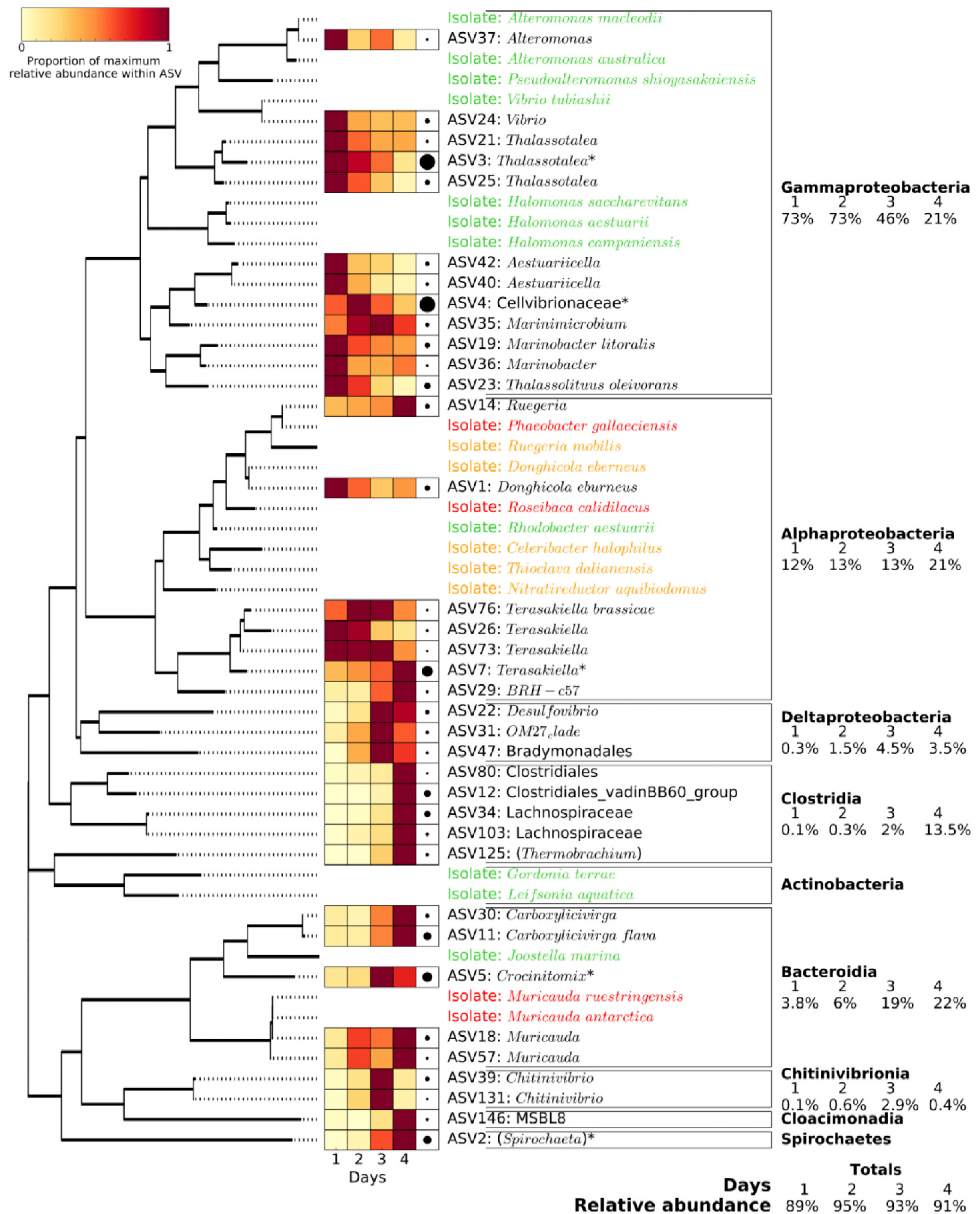


Figure 4. Phylogenetic analysis and relative abundance of the major 16S rRNA gene ASVs (*i.e.* with relative abundance above 0.5% in at least one of the four days) and bacterial isolates obtained at the end of the artificial selection experiment. Phylogenetic grouping is represented by a mid-point

rooted maximum likelihood phylogenetic tree. The 36 ASVs represented in the figure (out of the 6605 total ASVs detected) accounted for 92% of all 16S rRNA gene relative abundance. The heatmap represents the relative abundance of each ASV over the four days, with darker red showing the day at which the ASV showed maximum abundance. Black circles on the right of the heatmap represent the maximum relative abundance for that ASV amongst the entire community. The 20 isolates are coloured depending on their ability to grow on chitin and the monomer, GlcNAc (green), the GlcNAc only (orange), or neither (red).

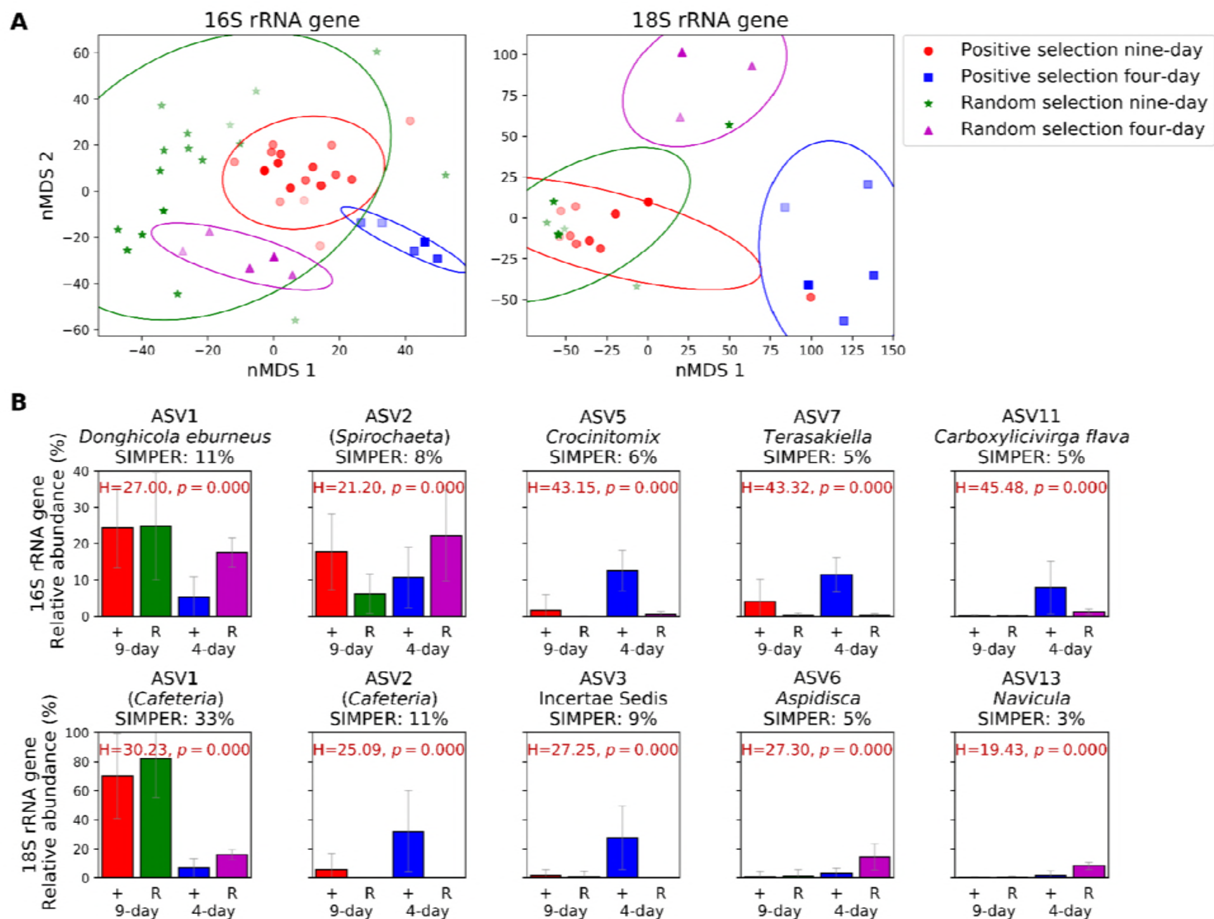


Figure 5. Microbial community variation over the entre artificial selection experiment. (**A**) nMDS plot showing Bray-Curtis distance of 16S (left) and 18S communities (right). Distance between the community composition obtained from nine-day (red circles) and four-day incubations (blue squares) of the positive selection, and nine-day (green stars) and four-day incubations (purple triangles) of the random controls are shown. Marker colour intensity correlates to generation number, where progressive darker colours represent later generations. Each point represents the mean of the three communities selected from one generation used to inoculate the following one. Ellipses show the mean plus the standard deviation of each group of samples. Stress values are 0.175 for the 16S rRNA gene and 0.063 for the 18S rRNA gene. (**B**) Five 16S (top panel) and 18S rRNA gene ASVs (bottom panel) that contributed the most towards community variations between the nine-day (generations 0-20) and four-day (generations 16-20) positive (+) and random (R)

selections according to SIMPER analyses. The percentage of variation to which each ASV contributes is indicated. ASVs were classified to the species level with the standard analysis pipeline using the SILVA database where possible. Names in brackets were not identifiable and were identified through a BLAST search of the NCBI database. Relative abundances and error bars shown are the mean and standard deviations of all generations within that treatment.

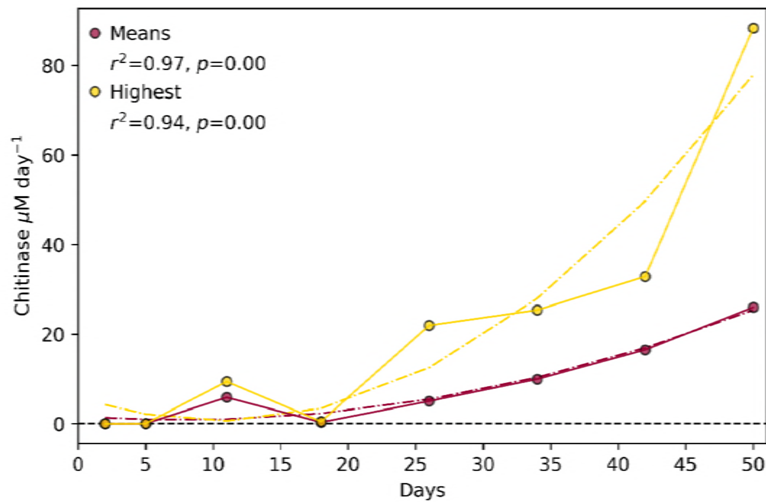


Figure 6. Chitinase activity of artificial selection experiment 2. Graph show the mean chitinase activity of the positive selection, from which the mean random selection was subtracted. The means of all communities within the generation (n=30; red) and those of only the three communities that were pooled for the inoculum of the next generation (yellow) are shown. The r^2 and p-values are for Pearson's correlation coefficients and lines of best fit (dotted lines) were determined using linear regression models within Pythons' statsmodels package.