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1   Adaptation to a limiting element involves mitigation of multiple  
2   elemental imbalances

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16  **This manuscript includes:**

17       Main Text  
18       Figures 1 to 3  
19       Tables 1 to 2  
20       Supplementary Files (1 Table; 2 Figures; 1 data file)

21

22

23 **Abstract:** About twenty elements underlie biology, and thus constrain biomass production.  
24 Recent systems-level observations indicate that altered supply of one element impacts the  
25 processing of most elements encompassing an organism (i.e., ionome). Little is known about the  
26 evolutionary tendencies of ionomes as populations adapt to distinct biogeochemical  
27 environments. We evolved the bacterium *Serratia marcescens* under five conditions (i.e., low  
28 carbon, nitrogen, phosphorus, iron, or manganese) that limited the yield of the ancestor  
29 compared to replete medium, and measured the concentrations and use efficiency of these five,  
30 and five other elements. Both physiological responses of the ancestor, as well as evolutionary  
31 responses of descendants to experimental environments involved changes in the content and use  
32 efficiencies of the limiting element, and several others. Differences in coefficients of variation in  
33 elemental contents based on biological functions were evident, with those involved in biochemical  
34 building (C, N, P, S) varying least, followed by biochemical balance (Ca, K, Mg, Na), and  
35 biochemical catalysis (Fe, Mn). Finally, descendants evolved to mitigate elemental imbalances  
36 evident in the ancestor in response to limiting conditions. Understanding the tendencies of such  
37 ionic responses will be useful to better forecast biological responses to geochemical changes.

38

### 39 **Introduction**

40 The lowest level of biological organization is represented by about twenty elements (Williams &  
41 Frausto da Silva 2005), and is referred to as the ionome, the entire suite of elements  
42 encompassing an organism (Salt et al. 2008). Some of these elements (i.e., carbon, hydrogen,  
43 oxygen) are necessary for the organic framework required for all major biomolecules (e.g.,  
44 carbohydrates, proteins, lipids) and are the most abundant bioelements. Others play key roles in  
45 the: structures of biomolecules (e.g., nitrogen, phosphorus, sulfur), maintenance of ionic balance  
46 (e.g., calcium, sodium, magnesium), and catalytic functions (e.g., iron, manganese). Elemental  
47 quotas of cells are under selection because perturbations to the supply or processing of an  
48 element (e.g., mutation in a transporter) impact growth and fitness (e.g., Jeyasingh & Weider

49 2007; Merchant & Helmann 2012). Biologists have traditionally focused on the elements that are  
50 most imbalanced between supply and biological demand to predict key biological process (e.g.,  
51 growth; van der Ploeg et al. 1999), from cells (Droop 1973) to ecosystems (Schindler 1977).

52 Because no element functions in isolation, efforts to characterize the effect of any one  
53 biogenic element quickly discovered the importance of elemental linkages in biology (e.g.,  
54 Redfield 1958; Sterner & Elser 2002). The ratios of abundant bioelements (e.g., C, N, and P)  
55 have been useful in exploring key ecological (e.g., Sterner et al. 1992), evolutionary (e.g., Kay et  
56 al. 2005) and eco-evolutionary processes (e.g., Jeyasingh et al. 2014). Several studies have also  
57 isolated the impact of elemental supply in the contents of key elements in biomass over micro-  
58 (Turner et al. 2017; Warsi & Dykhuizen 2017), and macro-evolutionary timescales (Baudouin-  
59 Cornu et al. 2001; Acquisti et al. 2009; Quigg et al. 2003). Typically, adaptation to a limiting  
60 element is thought to result in changes in use of that element by altering its efficiency, substituting  
61 it with another element with similar functions, or entirely dispensing it (reviewed in Merchant &  
62 Helmann 2012). Yet, such studies consider only a small subset of biogenic elements. A common  
63 assumption in such work is that information on the other (unmeasured) elements that are in the  
64 system is superfluous. While such assumptions (e.g., single-nutrient limitation) are due to  
65 mathematical and empirical obstacles, they must be revisited, particularly when models based on  
66 such assumptions perform poorly in describing nature (e.g., Sommer 1991; Harpole et al. 2011).  
67 Moreover, observations in the post-genomic era are illuminating considerable diversity in the  
68 physiological processing of a single element within and among species because they are  
69 quantitative traits controlled by numerous loci, and changes in the supply of a single element  
70 invoke system-wide physiological adjustments in the quotas of most, if not all elements found in  
71 an organism (i.e., its ionome; Baxter 2015; Huang & Salt 2016).

72 In addition to complexity at the physiological level highlighting the importance of  
73 information of most biogenic elements in a system under study, the geochemical conditions in  
74 each location is also heterogeneous, with the abundance of one element impacting the availability  
75 of multiple others (e.g., Gustafsson 2013). We know surprisingly little about the relevance of

76 geochemical heterogeneity to the biological processes that happen upon it, and may underlie the  
77 prevalence of nutrient co-limitation of ecological systems (Harpole et al. 2011; Fay et al. 2015;  
78 Browning et al. 2017). Another pressing motivation is to understand the biological relevance of  
79 anthropogenic changes to not only abundant (e.g., P, Elser & Haygarth 2020) but also trace (e.g.,  
80 Fe; Björnerås et al. 2017) bioelements (Kaspari 2021). For example, Peñuelas et al. (2022) found  
81 that while the use of elements involved in biochemical structure has changed between 10-(e.g.,  
82 C) and 80-(e.g., P) fold, the Anthropocene is characterized by over a 100-fold change in most  
83 metals involved in catalysis (e.g., Fe, Zn), with nickel (Ni) use having increased over a 1000-fold.  
84 Integrative, systems-level approaches will be required to forecast the biological responses to  
85 changes in the cycles of bioelements (e.g., Peñuelas et al. 2019; Bianchi 2021).

86         One way to gain a systems-level understanding of the relevance of any one element is to  
87 not only focus on the quotas of elements encompassing an individual, but also the use efficiency  
88 of an element. Defined as the amount of new biomass produced per unit element assimilated,  
89 nutrient use efficiencies (NUE; Vitousek 1982) are quantitative traits that impact growth at lower  
90 levels (e.g., organismal; Sherman et al. 2020) manifesting as biomass production and material  
91 fluxes at higher levels of organization (e.g., ecosystem; Fukushima & Matsushita 2021). Although  
92 we have known that changes in the NUE of a single element is associated with correlated  
93 changes in the NUEs of other elements (e.g., Jeyasingh et al. 2017; 2020), we have yet to  
94 capture the general tendencies of ionomes and ionome-wide NUEs as evolution proceeds in  
95 distinct biogeochemical environments (Sardans et al. 2021). Another way to explore the chemical  
96 system in the context of biology is to observe its behavior when known perturbations are applied,  
97 compared to some idealized environment (e.g., fastest growth; maximal yield). Such an exercise  
98 is similar to the approach used by ecologists to identify imbalances in elements between a  
99 consumer and its diet with simplifying assumptions regarding bioavailability (e.g., the trophic  
100 stoichiometric ratio; Filipiak & Weiner 2014). Comparing the quota of an element in the ideal (e.g.,  
101 fastest growth) condition with the quota of that element in a limiting (e.g., slower growth) condition  
102 provides information on the elements that are imbalanced, as well as the degree of imbalance.

103 Such information places ionome-wide data in the context of substantial modular information on  
104 the metabolism of each element (e.g., Kaim et al. 2013).

105 In this study, we first (i) tested predictions about the relationships between supply of  
106 various elements (i.e., C, N, P, Fe, Mn) and the growth of the cosmopolitan bacterial pathogen,  
107 *Serratia marcescens* (Grimont & Grimont 1978; Flyg et al. 1980). Compared to a replete medium  
108 (referred to as Full), we expected low supply of elements involved in biochemical building (i.e.,  
109 LowC, LowN, LowP) will result in larger growth and yield penalties than low supply of trace  
110 elements (i.e., LowFe, LowMn) that serve catalytic functions. We then (ii) tested the nutrient  
111 sparing hypothesis, which predicts that lineages acclimating or adapting to low supply of an  
112 element will increase their use efficiency for this element ( $NUE = \text{yield} \div \text{concentration of}$   
113  $\text{element}$ ). Next, we (iii) tested whether ionomes of descendants are more variable than the  
114 ancestor, and quantified ionic divergence across different environments. Furthermore, (iv) we  
115 predicted that trace elements involved in catalysis, with fewer loci directly involved in their  
116 processing, would be more prone to disruptions due to *de novo* mutations and vary more  
117 compared to bulk elements involved in building and balance of most, if not all, biochemicals that  
118 are under the control of multiple loci. Finally, (v) because we expected (and found, see below)  
119 ionomes in the limiting treatments (i.e., LowC, LowN, LowP, LowFe, LowMn) will differ from that  
120 of the ancestor in the fastest growing conditions (i.e., Full), we tested whether lineages evolve  
121 toward an ionome that is similar to that of the ancestor in the fastest growing condition using  
122 isometric log-ratio balances.

123

## 124 **Materials and Methods**

125 *Study organism:* We used a single clone of *Serratia marcescens* (DB 11; Flyg et al. 1980) as the  
126 ancestor. A few cells of a single clone frozen in glycerol was resuscitated overnight in LB medium  
127 to derive cells for the initiation of the experimental lineages. *S. marcescens* is an opportunistic  
128 bacterium that is ideal for experimental evolution because it can be grown at room temperature,  
129 thrives in a wide variety of conditions, and its cultures are difficult to contaminate with other

130 microbes. *S. marcescens* is a cosmopolitan and pathogenic bacterium capable of infecting  
131 several species (Grimont & Grimont 1978). It has been used extensively in experimental evolution  
132 studies and shown to evolve in response to various environmental conditions such as  
133 temperature (e.g., Ketola et al. 2013, Bruneaux et al. 2022) presence of predators (e.g., Friman et  
134 al. 2008) and competing bacterial species (e.g., Ketola et al. 2016).

135

136 *Growth and yield of the ancestor in the six nutrient supply conditions:* Growth measurements  
137 were initiated by pipetting 100  $\mu$ L of thawed stock of ancestor in 5mL of modified M9 full growth  
138 medium. After 24 h, 10  $\mu$ L of this culture was transferred into each well of the 100-well Bioscreen  
139 plates containing 400  $\mu$ L of media from each of the six media (Table S1). Each treatment was  
140 replicated 16 times, for a total of 96 measurements. Growth was measured in a Bioscreen  
141 spectrophotometer (Growth Curves AB Ltd. Helsinki, Finland) at 600 nm in 5 min intervals for 3-7  
142 days until growth had clearly reached maximum yield in all wells. Maximal growth rate and yield  
143 were analyzed from the optical density (OD) measurements with a MATLAB (version 2008b; Math  
144 works Inc., Natick, MA, USA) script that fits linear regressions into ln-transformed population  
145 growth data consisting of 30-datapoint sliding time window (see Ketola et al. 2013). Maximum  
146 growth is found within the window with the steepest linear regression. Yield was given by the  
147 maximal average OD among the sliding windows. Analysis of variance, followed by Tukey's post  
148 hoc tests were used to test whether the environment had significant effects of growth and yield.

149

150 *Experimental evolution:* The evolution experiment was initiated from the ancestral strain that was  
151 revived from a stock maintained at -80°C by inoculating 100  $\mu$ L into 10 mL of modified M9 growth  
152 medium and grown at ~20°C for 74h. Ten  $\mu$ L of this preculture was inoculated into 5 mL of each  
153 of the six growth media (Table S1) in 15 mL culture tubes. Each treatment was replicated 10  
154 times (i.e., 10 lineages evolving in each of the 6 treatments) and maintained in static cultures at  
155 room temperature. After thoroughly homogenizing, 5  $\mu$ L of each culture was transferred to fresh  
156 respective media every 24h for 29 days. Note that this transfer regime is meant to sample the

157 most common life-history strategy in the population. Optical densities (OD) of 400  $\mu$ L sample of  
158 bacterial cultures grown overnight were measured daily on a Bioscreen spectrophotometer  
159 (Growth Curves AB Ltd. Helsinki, Finland) at 600 nm in 5 min intervals for the entire duration of  
160 the experiment. The mean of the first 3 OD measurements was used in calculation of  
161 accumulated generations as follows:  $\log_2 [(OD \text{ day } x \div OD \text{ day } x-1) \times (\text{inoculum size} \div \text{culture}$   
162  $\text{volume})]$ .

163 The purity of all cultures was verified using species-specific 16S rRNA markers and  
164 confirmed to be *Serratia marcescens*. Bacterial cells were harvested from fresh cultures by  
165 centrifugation at 4000 x g for 10 min. Bacterial genomic DNA was extracted using the Wizard  
166 Genomic DNA Purification Kit (Promega, USA). Amplifications of the bacterium-specific 16S  
167 rRNA gene region were performed using universal primers 799f and 1492r. PCR reactions were  
168 performed in a total volume of 20  $\mu$ l containing 4  $\mu$ l of 5X green reaction buffer, 0.2  $\mu$ l GoTaq  
169 DNA polymerase (5 U/ $\mu$ l), 1  $\mu$ l of 0.5  $\mu$ M forward primer, 1  $\mu$ l of 0.5  $\mu$ M reverse primer, 2  $\mu$ l of 0.2  
170 mM dNTPs, 10.8  $\mu$ l of distilled H<sub>2</sub>O and 1  $\mu$ l of genomic DNA. PCR reactions were performed on  
171 a BioRad 1000C thermal cycler, under the following conditions: 95°C for 30 minutes, followed by  
172 30 cycles at 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for one minute, and a final  
173 extension at 72°C for 5 minutes. Five microliters of the PCR products were run on a 1.5%  
174 agarose gel to verify correct amplification. The PCR products were purified using 10 U of  
175 Exonuclease I and 1 U of Fast APTM Thermosensitive Alkaline Phosphatase (Fermentas GmbH,  
176 Germany) for 15 minutes at 37°C, followed by enzyme inactivation for 15 minutes at 85°C. The  
177 purified PCR products were then sequenced with the same primers used in amplification using  
178 Big Dye Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems). Briefly, each 20  $\mu$ l  
179 sequencing reaction mixture contained 1  $\mu$ l of PCR amplicon, 0.16  $\mu$ M of either forward or  
180 reverse PCR primer, 0.5  $\mu$ l of BigDye Ready Reaction Mix, and 1 X sequencing buffer. The  
181 sequencing reaction conditions were as follows: 30 cycles of denaturing at 96°C for 10 seconds,  
182 annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. The sequencing products  
183 were purified using ethanol/EDTA/sodium acetate precipitation. Sequencing was performed on an



184 ABI 3130xl 16-capillary automated genetic analyzer. The sequences were compared with the  
185 NCBI database through BLAST searches to confirm species identity as *Serratia marcescens*  
186 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

187

188 *Growth and yield:* At the conclusion of experimental evolution trials, 8 clones were extracted from  
189 eight of the populations in each evolutionary treatment. Clones were isolated by dilution plating  
190 on nutrient broth agar and grown overnight into high OD in 1 mL of the respective medium where  
191 the population had been evolving. The clone cultures were frozen in a 1:1 ratio with 80% glycerol  
192 on Bioscreen C 100-well plates and stored at -80°C. Two clones from each of the 8 populations,  
193 from all 6 evolutionary treatments were randomized in a 100 well Bioscreen plate which included  
194 four ancestral clones ( $2 \times 8 \times 6 = 96 + 4 = 100$ ). The remaining 6 clones were treated similarly, for  
195 a total of four full plates (i.e., 400 clones). This frozen clone library enabled easy and fast growth  
196 measurements from frozen stocks using a cryo-replicator system (Duetz et al. 2000; Ketola et al.  
197 2013; 2016). Growth measurements were initiated by cryo-replicating the frozen clones into wells  
198 of a Bioscreen plate filled with 400  $\mu$ L of full growth medium. After 24 h, 10  $\mu$ L of each clone  
199 culture was transferred into each well of the 100-well Bioscreen plates containing 400  $\mu$ L of  
200 media from respective treatments. All four replicate clone plates were measured in all six growth  
201 media. Bacterial growth was measured in a Bioscreen spectrophotometer for 3-7 days until  
202 growth reached maximum yield in all wells (see above). Analysis of variance, followed by Tukey's  
203 post hoc tests were used to test for significant differences in growth and yield due to treatment  
204 and evolution.

205

206 *Ionomics:* For ionomics analyses, one clone from each of five populations evolving in each of the  
207 six media were randomly chosen. The clones were precultured by inoculation of 100  $\mu$ L of frozen  
208 culture into 1 mL of the modified M9 full medium overnight. Preculture was added into 250 mL of  
209 growth media in 500 mL Corning polycarbonate Erlenmeyer flasks (Corning Inc. New York,  
210 NY,USA) placed on a shaker at  $-20^{\circ}\text{C}$  and grown for 12h. This incubation time was chosen

211 based on pilot data to ensure that all cultures were in log phase growth at the time of harvesting  
212 (i.e., before the Full treatment reached K). While we acknowledge that population sizes of each  
213 treatment will be at different positions relative to K (with Full closest to, and LowC farthest from;  
214 Fig. 1), the ionic comparisons of interest in this paper is between the ancestor and  
215 descendants in identical conditions, minimizing any growth phase differences impacting the  
216 ionome. All clones were cultured in the medium they had been evolving in. For each clone, a  
217 replicate ancestral clone was cultured in the respective medium. The culture was then centrifuged  
218 (Sorvall RC-6+, Thermo Scientific, Waltham, MA, USA; 4570 x g, 15 min, 4°C), the supernatant  
219 was discarded and the pellet was diluted in 7.5 mL of autoclaved distilled water in 15 mL tubes,  
220 which were further centrifuged (Megafuge 1.0 R, Thermo Scientific, Waltham, MA, USA; 6000 rcf,  
221 20 min, 4°C). After removal of supernatant, the samples were frozen at -20°C until elemental  
222 analyses.

223         Samples were dried at 60°C for 72h, and subsamples of known mass (to the nearest µg;  
224 XP2U, Mettler Toledo, Columbus, OH, USA) were analyzed for carbon and nitrogen content using  
225 an automated analyzer (varioMicro Cube; Elementar Americas, Mt. Laurel, NJ, USA). Another  
226 subsample of known mass was rinsed in oxalate to minimize elements adsorbed to cells (Hassler  
227 & Schoemann 2009), digested for 24 h in 70% trace metal grade HNO<sub>3</sub> in 15 mL metal-free  
228 polypropylene tubes and injected into an inductively-coupled plasma optical emission  
229 spectrometer (ICP-OES, Thermo Scientific icAP 7400, Waltham, MA, USA) to quantify all other  
230 elements in the *Serratia* ionome. ICP-OES analysis was validated and calibrated with aqueous  
231 multi-element external standard reference solutions (CPI International, Santa Rosa, CA, USA)  
232 and an in-line internal standard of Yttrium (CPI International, Santa Rosa, CA, USA) to correct for  
233 instrument drift or potential matrix effects. When the elemental concentrations were within the  
234 range of blank controls, they were considered to be at the detection limit of the instrument and  
235 excluded from further analysis. This resulted in 10 elements which were above LOD in all  
236 samples. We calculated nutrient use efficiencies (NUE) of each of these 10 elements and

237 compared differences among environments and evolution using MANOVA, followed by Tukey's  
238 post hoc tests.

239 Differences in ionic composition across nutrient limitation treatments were assessed  
240 using isometric log ratios (ILRs). Raw elemental data were first subdivided into linearly  
241 independent ratios (or balances) by splitting the dataset sequentially into smaller parts. For  
242 instance, a dataset containing measurements of C, N, and P may be split into two dual ratios  
243 (e.g., C:P and N:P). ILRs were then calculated as:

$$244 \quad \text{ILR} = \text{SQRT} \left( \frac{r}{r+s} \right) \ln \left[ \frac{g(c^+)}{g(c^-)} \right] \quad (\text{Egozcue and Pawlowsky-Glahn 2005})$$

245 where  $r$  and  $s$  represent the number of elements on the left- and right-hand side of the ratio and  
246  $g(c^+)$  and  $g(c^-)$  minus represent the geometric mean of elemental percentages on the left- and  
247 right-hand side of the ratio, respectively. Once calculated, we ran a principal components analysis  
248 (PCA) on this collection of ratios, and total ionic imbalance for each form of nutrient limitation  
249 were diagnosed through comparisons to ancestral isolates using pairwise 95% confidence  
250 intervals of Aitchison distances (i.e., centroid  $\pm$  95% CI overlap of unbiased ILRs). As ILR  
251 ordinations can be difficult to interpret, we examined imbalances for individual elements in each  
252 limitation treatment using concentration ratios of an element under nutrient limitation standardized  
253 to the concentration of this element in the ancestral strain growing in the Full medium (with fastest  
254 growth and highest yield), where imbalances of a given element are indicated by 95% CIs not  
255 overlapping optimum threshold values of 1 (Parent et al. 2020). Differences in individual  
256 elemental concentrations between ancestral and descendent lineages are likewise indicated when  
257 confidence intervals of these groups do not overlap.

258

## 259 **Results**

260 Maximal growth rate ( $r_{\max}$ ) of the ancestor were affected by the nutrient environment (Fig. 1;  
261  $F_{5,90} = 4.08$ ;  $P = 0.02$ ), with Tukey's HSD post hoc tests revealing that  $r_{\max}$  in LowP was different  
262 from Full and LowFe. No other treatments were different from each other. Yield was also affected  
263 by treatment ( $F_{5,90} = 39.22$ ;  $P < 0.0001$ ), with post hoc tests revealing four homogenous subsets

264 (i.e., treatments within a subset do not significantly differ from each other): (i) Full, LowMn, (ii)  
265 LowFe, (iii) LowP, and (iv) LowN, LowC. We considered the Full treatment as the unlimited,  
266 reference treatment to contrast with other treatments.

267 All lineages were evolved for ~285 generations (mean= 285.91; SD= 0.73). Descendants  
268 differed from the ancestor in both  $r_{\max}$  and yield depending on the nutrient supply environment, as  
269 indicated by the posthoc results denoted by stars above boxplots (Fig. 2). Compared to the  
270 ancestor, descendants exhibited lower  $r_{\max}$  in the LowN, LowFe, LowMn, and Full treatments but  
271 did not significantly differ in the LowC and LowP treatments ( $F_{5,87}= 21.51$ ;  $P<0.0001$ ). Yield of  
272 descendants were significantly lower compared to the ancestor in the LowC, LowN, and LowP  
273 treatments, as indicated by posthoc tests and denoted by stars above boxplots ( $F_{5,82}= 53.39$ ;  
274  $P<0.0001$ ). Yield did not significantly diverge in the LowFe, LowMn, and Full treatments.  
275 Significant differences in both  $r_{\max}$  and yield between ancestor and descendants were observed  
276 only in the LowN treatment.

277 *Serratia* from different treatments occupied different regions of ionic space (Fig. S1).  
278 To test whether such shifts are driven by ionome-wide nutrient sparing, we performed a MANOVA  
279 on the nutrient use efficiencies of the ten measured elements (NUE; yield  $\div$  concentration of  
280 element in *Serratia*). The test was significant and revealed interactive effects of environment and  
281 evolution on the NUE of C, N, Fe, Ca, Na, and S (Table 1). Both environment and evolution  
282 independently impacted the use efficiency of P and Mg, while Mn and K use efficiencies were  
283 impacted by the environment alone. Univariate analyses (Fig. S2) illuminated substantial  
284 differences in the NUE of elements due to the environment, evolution, and their interaction.  
285 Importantly, the shifts in NUE were not only related to the element that was experimentally  
286 manipulated (e.g., P) but also several others. For example, although LowP conditions caused a  
287 divergence in the NUE for P (Fig. S2a), it also caused a divergence in the use efficiencies of C  
288 (Fig. S2a), S, and Ca (Fig. S2b).

289 Coefficients of variation (CV) measurements show that highly abundant elements  
290 involved with biomass construction (C, N, S) were less variable across all lineages/limitation

291 treatments with the exception of P, which is also involved in catalysis. Elements involved with  
292 charge balance (Na, Ca, K, Mg) were the second most variable, and trace catalytic elements (Fe,  
293 Mn) varied the most (Table 2a). CV values indicate that descendant lineages were more variable  
294 than ancestors, except under LowFe (Table 2b).

295           Ionomic variation appeared to be related to evolutionary changes in elemental use  
296 intended to balance cellular concentrations of a given limiting element through correlated shifts in  
297 a few elements in LowC, LowN and Low P treatments, and ionome-wide adjustments in LowFe  
298 and LowMn treatments (Fig. 3). Descendants evolved in the Full treatment exhibited similar  
299 ionomes to ancestral controls (i.e., confidence interval for all elements overlap the dashed vertical  
300 line in Fig. 3a). In all other treatments (Fig. 3b-f), descendants evolved unique ionomic responses  
301 to individual forms of limitation compared to the ancestor (i.e., there is no overlap in the  
302 confidence intervals of some elements). For example, in the LowC treatment (Fig. 3b) the C  
303 content bar of the ancestor does not overlap the dashed line, while that of the descendants does.  
304 More apparently, Na content of the ancestor in LowC conditions is significantly higher than the Na  
305 content of the ancestor in the Full treatment (represented by the dashed line) because confidence  
306 intervals do not overlap the dashed line. The adjacent bar indicating the Na content of  
307 descendants evolved in LowC showed an overlap with the dashed vertical line, indicating a  
308 mitigation of excess Na over evolutionary time. Note also that all such imbalances were not  
309 mitigated. For example, both ancestor and descendants in LowC conditions contained more P  
310 than the ancestor in the Full treatment. Several such differences were also evident in other  
311 treatments (Fig. 3c-f), indicating ionome-wide adjustments.

312

### 313 **Discussion**

314 Significant treatment effects on growth parameters of the ancestor indicate that the conditions  
315 were suitable for the culture of *Serratia*, and that the chemical manipulations of the culture  
316 medium were physiologically relevant for *Serratia*. Consistent with prior observations on several  
317 taxa, including *Serratia* (Poole & Braun 1988; Angerer et al. 1992; Kuo et al. 2013; Pittman et al.

318 2015), the greatest impact on yield was due to lower energy (glucose) supply, followed by lower  
319 supplies of bulk elements (N, P), while impacts of lower trace metal supplies were relatively  
320 muted (Fig. 1). As discussed in Warsi et al. (2018), relatively little is known about the adaptive  
321 responses of microbes to limitation by metals, which impact different physiological components  
322 compared to macronutrients (e.g., C, N, P). Growth curves from six different conditions indicate  
323 distinct impacts on physiology (Fig. 1a), with limited supply of elements involved in biochemical  
324 building (C, N, P) decreasing yield to a greater degree compared to elements involved in  
325 biochemical catalysis (Fe, Mn). Importantly, evolutionary diversification in growth rate and yield  
326 depended on the environment, with only lineages evolving under N scarcity altering both growth  
327 and yield (Fig. 2). In all other treatments, either growth or yield diverged. Treatments that had  
328 large impacts on the yield of the ancestor (i.e., LowC, LowN, LowP; Fig. 1b) resulted in significant  
329 reduction in yield of the descendants (Fig. 2b). On the other hand, treatments that caused  
330 divergence in growth rate (i.e., LowFe, LowMn, Full; Fig. 2a) were conditions that had longer  
331 period of fast growth prior to entering the stationary phase (Fig. 1a). The LowN treatment belongs  
332 to both of these groups, and accordingly, evolutionary effects can be found in both traits when  
333 evolved in N limitation (Fig. 2). Together, these observations indicate that selection on the shape  
334 of the growth curve depends on the biogeochemical environment. Because such adaptation is not  
335 only due to loci underlying elemental use, but also loci controlling other covarying traits (e.g., size;  
336 Gounand et al. 2016) further studies exploring such interactions (e.g., by studying strains of  
337 different sizes or controlling renewal rate in chemostats) are needed to explain the distinct growth  
338 curves in response to changes in the supply of various elements.

339 Note that values of the growth parameters ( $r$ ,  $K$ ) were lower in the descendants  
340 compared to the ancestor. Although counterintuitive, it has been observed in experimental  
341 evolution studies (e.g., Ketola et al. 2004; Lenski 2010) as discussed in Kokko (2021).  
342 Specifically, although theory predicts that natural selection should increase mean fitness, fitness  
343 proxies such as growth can become uncoupled from fitness. For example, it is plausible that the  
344 abstracted lab conditions, with different selection pressures compared to the environment from

345 which the ancestor was isolated, selection can act to reduce both growth and yield to match  
346 resource renewal cycle and abundance of resources.

347         While growth is a classical trait of interest to evolutionary biologists, evolution in growth  
348 must be associated with adjustments in the materials required for growth, with important  
349 ecological impacts. However, because the ancestor adapted to experimental conditions by  
350 decreasing values of classical fitness proxies (i.e., growth rate, yield), adaptive inferences  
351 regarding the shifts in NUEs is not straightforward. For example, direct selection for rapid growth  
352 would enable us to test predictions arising from the growth rate hypothesis (e.g., Isanta-Navarro  
353 et al. 2022). Regardless, the main inferences arising from observations on NUEs across the  
354 ionome is that adaptation to limiting supply of an element often involves shifts in the use of not  
355 only the limiting element (as posited by the nutrient sparing hypothesis), but also correlated shifts  
356 in multiple other elements (Table 1). For example, in LowP conditions, significant divergence in  
357 NUE was observed for not only P, but also C, S, and Ca (Fig. S2). We have little theoretical  
358 guidance regarding the ionomic responses of organisms and represents an important frontier.  
359 Understanding ionome wide shifts in NUEs should have important ecological and evolutionary  
360 implications because it represents a change in the biogeochemical niche (Penulas et al. 2019).  
361 While much remains to be understood about the ecological relevance of ionomes (Jeyasingh et  
362 al. 2017; Kaspari 2021; Hofmann et al. 2021), we do know that differences in the NUE of multiple  
363 elements impact trophic transfer (e.g., Jeyasingh et al. 2020), and can alter the geochemical  
364 environment for subsequent generations (e.g., San Roman & Wagner 2018).

365         Although we are far from a mechanistic explanation of observations reported herein, the  
366 data suggest that quotas of different elements may evolve at different rates. The experimental  
367 evolution design employed here allowed us to ask whether *de novo* mutations impact the quotas  
368 of all elements similarly. First, we found that descendant ionomes exhibited greater variation than  
369 the ancestor in all environments besides LowFe (Table 2), indicating the key role of mutations in  
370 generating ionomic diversity. Moreover, ionomes of descendants diverged more from that of the  
371 ancestor in environments that did not constrain yield (i.e., LowFe, LowMn, and Full), while

372 ionomes of descendants in environments that had large effects on yield (i.e., LowC, LowN, LowP)  
373 exhibited ionic overlap (Fig. S1) suggesting that greater number of cell divisions in the Full,  
374 LowFe, and LowMn treatments allowed greater divergence of populations.

375           Nevertheless, ionic variation was not equally partitioned among the measured  
376 elements either in the acclimatory responses of the ancestor to different treatments or in the  
377 evolutionary responses of descendants. Elements involved in biomass construction (C, N, P)  
378 were less variable compared to elements involved in ionic balance (Na, Ca, K, and Mg), and  
379 biochemical catalysis (Fe and Mn), which varied the most. Ionomes of descendants in the Full  
380 medium diverged considerably in elements serving catalytic functions although such imbalances  
381 arose in a lineage-specific manner (Table 2). Interestingly, elements involved in biochemical  
382 building and balance were not as highly imbalanced or variable as trace metals among replicate  
383 descendant lineages. This observation suggests that evolutionary tendencies of ionomes may not  
384 be equal in all dimensions. One hypothesis to explain this pattern arises from the nature of  
385 genomic architecture, where some elements (e.g., Fe, Mn) are controlled by fewer loci than  
386 others (e.g., C, N, P) and more prone to be disrupted by *de novo* mutations. In other words, the  
387 number of loci impacting proteins that control the intake, metabolism, and storage differ among  
388 elements. For example, of the 4622 proteins coded for by the *Serratia* genome (NCBI ID 1112),  
389 126 are involved in processing of P, while 26 are involved with Fe, and only one for Ni. Thus, a  
390 mutation at a locus underlying Ni processing could have a much higher impact on the  
391 concentration of Ni in the cell, compared to the impact of a mutation at a locus underlying P  
392 processing on the P content of a cell.

393           Comparison of ancestral ionomes in the Full medium with that of ancestral and  
394 descendant ionomes (i.e., imbalance ratios) in the various limitation treatments (Fig. 3) provided a  
395 window into the system-wide adjustments made in response to selection specific to each  
396 biogeochemical condition. While multivariate ionic imbalance ratios in all treatments differed  
397 from that of the optimally growing ancestor, significant divergence of elemental imbalances  
398 across the ionome was observed only in LowFe and LowMn treatments (Fig. 3e, f). In both cases,



399 the elemental imbalance ratio was lower in descendants than in the ancestor acclimating to the  
400 same environment, suggesting some adaptive value in mitigating elemental imbalances. Such  
401 mitigation did not involve the element in limiting supply (i.e., Fe or Mn), rather descendants in  
402 LowFe decreased their Na imbalance and those in LowMn treatments decreased their Fe  
403 imbalance compared to the ancestor. As LowFe organisms also increased their Mn  
404 concentrations, these results suggest substitution of these two adjacent elements in the periodic  
405 table (Fitsanakis et al. 2010). Clearly, there are more such complex interactions in the data  
406 reported herein, as well as those typical of ionomic studies. For example, Eide et al. (2005)  
407 quantified the ionomes of over 200 yeast genotypes and found that mutations impacted the  
408 quotas of multiple elements, and most genotypes occupied unique locations in ionomic space.  
409 Placing such ionome-wide observations in the context of our understanding about the evolution of  
410 elemental quotas and use indicates that a focus on unitary elements, while illuminating the  
411 biochemical mechanisms (e.g., Casey et al. 2016), may miss substantial shifts in the quotas and  
412 use of other elements, which must impact the chemistry of the environment, potentially altering  
413 multifarious selection on subsequent generations.

414 Ionomic changes emerge from myriad genomic, anatomical, and physiological  
415 adjustments. While mapping such responses is beyond the scope of any one study, general  
416 inferences regarding the evolution of ionomes can guide exploration of biochemical mechanisms  
417 as well as ecological consequences (Jeyasingh et al. 2014; Penuelas et al. 2019). We observed  
418 (Table 2) that elements involved in biochemical catalysis (e.g., Fe, Mn) are more variable and  
419 evolutionarily labile than elements involved in biochemical balance (e.g., Ca, Na) and biochemical  
420 building (e.g., C, N). Lability of metal catalysts is particularly noteworthy for the study species, the  
421 pathogenic *S. marcescens*, because metals play key roles in virulence (Palmer & Skaar 2016).  
422 More specifically, our observations indicate that it is possible that metal quotas change as a  
423 population adapts to differences in the supply of other elements, thereby increasing the likelihood  
424 of virulence. Although the mechanism of virulence may not change (e.g., a metal in a pathogen  
425 enzyme oxidizing host integument, Aachmann et al. 2012), allocation of metals to such virulence-

426 relevant machinery may be sensitive to acclimatory or adaptive adjustments in response to  
427 limitation of another element. Consequently, measuring one or a small subset of elements to  
428 understand any biological process is bound to ignore a substantial proportion of underlying  
429 mechanisms. Observations in this study indicate that adaptation to limitation of a particular  
430 element involves readjustment of multiple other elements. Understanding the general tendencies  
431 of such rearrangement should reveal a systems-level picture of the dynamic interactions among  
432 genetics, traits, and the environment. More generally, this study highlights the utility of  
433 observations at the interface of inorganic chemistry and biology (e.g., Williams & Rickaby 2012) in  
434 advancing ecological and evolutionary theory, although much work remains. Placing our modular  
435 understanding of bioelements in systemic context, as attempted here, may be useful in  
436 forecasting biological phenomenon using chemical information – a central challenge in the  
437 Anthropocene characterized by rapid changes of large magnitude in the inorganic chemistry of  
438 the biosphere.

439

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448

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618 **Figures and Tables**

619 **Figure 1.** (a) Example growth curves of the ancestral *Serratia* strain in the six experimental  
620 conditions over the first 48h based on optical density measurements every 5 minutes at 600 nm.  
621 Curves depict averaged values of replicate measurements. Similar growth data over longer time  
622 periods were used to obtain yield and growth rate estimates (see methods). The vertical line at  
623 the 24h mark depicts the time at which cultures were renewed during the experimental evolution  
624 study. (b) Yield and growth rate ( $r_{\max}$ ) of the ancestor in the six nutrient supply treatments. Boxes  
625 indicate 1st and 3rd quartiles, with the line representing the median, while the whiskers indicate  
626 the maximum and minimum observed values. Dots represent outliers.

627

628 **Figure 2.** Differences in (a) growth rate ( $r_{\max}$ ) and (b) yield between ancestor and descendants in  
629 the six nutrient supply treatments at the end of the 28d experimental evolution study. Stars  
630 indicate significant post hoc differences between ancestor and descendant within each medium.  
631 Boxes indicate 1st and 3rd quartiles, with the line representing the median, while the whiskers  
632 indicate the maximum and minimum observed values. Dots represent outliers. Shaded boxes are  
633 ancestors, open boxes are descendants.

634

635 **Figure 3.** Ionomic elemental imbalance ratios in each of the six treatments: (a) Full, (b) LowC, (c)  
636 LowN, (d) LowP, (e) LowFe, (f) LowMn. Univariate imbalances for individual elements are  
637 expressed as concentration ratios relative to ancestral optimally growing phenotypes where 1 is  
638 perfectly balanced (dashed vertical line),  $<1$  is nutrient limited and  $>1$  is nutrient surplus. Error  
639 bars for each value represent 95% confidence intervals (CI's) where error bars not overlapping  
640 optimal balance thresholds indicate significant imbalances and nonoverlapping bars between  
641 ancestral and descendant lineages indicate differential responses to limitation. Multivariate  
642 ionomic balance differences were determined using Aitchisonian distances from optimally  
643 growing phenotypes and are denoted with a \* for a given treatment. Mean  $\pm$  95% CI's are given

644 for ancient (A) and descendant (D) lineages where higher values correspond to greater deviation  
645 from optimal phenotypes (centered at zero). Separate means are given for A & D lineages for a  
646 given limitation treatment only when they differ significantly from one another.

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663 **Table 1.** Effects of environment (LowC, LowN, LowP, LowFe, LowMn, Full) and evolution  
664 (Ancestor, Descendant) on the nutrient use efficiency (NUE= yield ÷ concentration of element in  
665 *Serratia*) of the 10 elements measured (see Figure 3). The overall MANOVA model was  
666 significant (Pillai's Trace;  $F_{Env} = 16.14$ ,  $df = 50, 215$ ,  $P < 0.0001$ ;  $F_{Evo} = 18.04$ ,  $df = 10, 39$ ,  $P < 0.0001$ ;  
667  $F_{Env \times Evo} = 2.61$ ,  $df = 50, 215$ ,  $P < 0.0001$ ).

668

Source	Dependent Variable (NUE)	df	F	Sig.
Environment	C	5	446.78	<0.000
	N	5	278.45	<0.000
	P	5	29.64	<0.000
	Fe	5	120.69	<0.000
	Mn	5	70.49	<0.000
	Ca	5	77.33	<0.000
	K	5	5.41	0.001
	Mg	5	81.17	<0.000
	Na	5	154.30	<0.000
	S	5	202.11	<0.000
Evolution	C	1	127.29	<0.000
	N	1	103.29	<0.000
	P	1	12.23	0.001
	Fe	1	3.07	0.086
	Mn	1	.07	0.792
	Ca	1	26.90	<0.000
	K	1	.01	0.895
	Mg	1	47.27	<0.000
	Na	1	6.97	0.011
	S	1	75.75	<0.000
Env * Evo	C	5	13.53	<0.000
	N	5	7.73	<0.000
	P	5	.69	0.633
	Fe	5	2.75	0.029
	Mn	5	.00	1.000
	Ca	5	6.85	<0.000
	K	5	.48	0.786
	Mg	5	1.28	0.288
	Na	5	6.15	<0.000
	S	5	8.35	<0.000

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671 **Table 2.** Functional and evolutionary ionomic variation. Coefficients of elemental variation (CVs)  
 672 are shown separately for (a) elemental functional groups averaged across treatments and (b)  
 673 elemental differences between the ancestor (A) and descendent (D) lineages averaged across  
 674 elements.

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(a) Function	Element	CV (%)	(b) Lineage	CV (%)
Building	C	4.8	Full A	13.8
	N	6.5	Full D	73.9
	P	21.0	LowC A	22.1
	S	8.3	LowC D	29.6
Balance	Ca	21.6	LowN A	21.6
	K	35.1	LowN D	77.9
	Mg	61.7	LowP A	10.8
	Na	70.7	LowP D	24.1
Catalysis	Fe	101.8	LowFe A	15.2
	Mn	129.4	LowFe D	11.7
			LowMn A	17.0
			LowMn D	21.7

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