JYX JYVÄSKYLÄN YLIOPISTO UNIVERSITY OF JYVÄSKYLÄ

This is a self-archived version of an original article. This version may differ from the original in pagination and typographic details.

Author(s): Jeyasingh, Punidan D.; Sherman, Ryan E.; Prater, Clay; Pulkkinen, Katja; Ketola, Tarmo

Title: Adaptation to a limiting element involves mitigation of multiple elemental imbalances

Year: 2023

Version: Accepted version (Final draft)

Copyright: © Authors 2023

Rights: CC BY 4.0

Rights url: https://creativecommons.org/licenses/by/4.0/

Please cite the original version:

Jeyasingh, P. D., Sherman, R. E., Prater, C., Pulkkinen, K., & Ketola, T. (2023). Adaptation to a limiting element involves mitigation of multiple elemental imbalances. Journal of the royal society interface, 20(198), Article 20220472. https://doi.org/10.1098/rsif.2022.0472

Adaptation to a limiting element involves mitigation of multiple elemental imbalances

Punidan D. Jeyasingh[1,2], Ryan E. Sherman[2], Clay Prater[2], Katja Pulkkinen[1], Tarmo Ketola[1]

[1] Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FI-40014, Finland.

[2] Department of Integrative Biology, Oklahoma State University, 501 Life Sciences West, Stillwater, OK 74078. U.S.A. Email: puni.jeyasingh@okstate.edu

Author Contributions: PDJ, KP, and TK conceived, designed, and conducted the experiments. RES generated ionomic data, that was analyzed by CP. PDJ interpreted data and wrote the manuscript with input from all authors.

Article Type: Research Article.

Competing Interest Statement: Authors declare no competing interests.

Keywords: ecological stoichiometry, experimental evolution, ionomics, nutrient use efficiency, *Serratia*.

Running title: Eco-evolutionary ionomics

This manuscript includes:

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

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

Introduction

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

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

The purity of all cultures was verified using species-specific 16S rRNA markers and confirmed to be *Serratia marcescens*. Bacterial cells were harvested from fresh cultures by centrifugation at 4000 x g for 10 min. Bacterial genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). Amplifications of the bacterium-specific 16S rRNA gene region were performed using universal primers 799f and 1492r. PCR reactions were performed in a total volume of 20 µl containing 4 µl of 5X green reaction buffer, 0.2 µl GoTaq DNA polymerase (5 U/µl), 1 µl of 0.5 µM forward primer, 1 µl of 0.5 µM reverse primer, 2 ul of 0.2 mM dNTPs, 10.8 µl of distilled H2O and 1 µl of genomic DNA. PCR reactions were performed on a BioRad 1000C thermal cycler, under the following conditions: 95°C for 30 minutes, followed by 30 cycles at 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for one minute, and a final extension at 72°C for 5 minutes. Five microliters of the PCR products were run on a 1.5% agarose gel to verify correct amplification. The PCR products were purified using 10 U of Exonuclease I and 1 U of Fast APTM Thermosensitive Alkaline Phosphatase (Fermentas GmbH, Germany) for 15 minutes at 37°C, followed by enzyme inactivation for 15 minutes at 85°C. The purified PCR products were then sequenced with the same primers used in amplification using Big Dye Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems). Briefly, each 20 µl sequencing reaction mixture contained 1 µl of PCR amplicon, 0.16 µM of either forward or reverse PCR primer, 0.5 µl of BigDye Ready Reaction Mix, and 1 X sequencing buffer. The sequencing reaction conditions were as follows: 30 cycles of denaturing at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. The sequencing products were purified using ethanol/EDTA/sodium acetate precipitation. Sequencing was performed on an

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

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

Ionomics: For ionomics analyses, one clone from each of five populations evolving in each of the six media were randomly chosen. The clones were precultured by inoculation of 100 µL of frozen culture into 1 mL of the modified M9 full medium overnight. Preculture was added into 250 mL of growth media in 500 mL Corning polycarbonate Erlenmeyer flasks (Corning Inc. New York, NY,USA) placed on a shaker at ~20ºC and grown for 12h. This incubation time was chosen

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

Samples were dried at 60ºC for 72h, and subsamples of known mass (to the nearest µg; XP2U, Mettler Toledo, Columbus, OH, USA) were analyzed for carbon and nitrogen content using an automated analyzer (varioMicro Cube; Elementar Americas, Mt. Laurel, NJ, USA). Another subsample of known mass was rinsed in oxalate to minimize elements adsorbed to cells (Hassler & Schoemann 2009), digested for 24 h in 70% trace metal grade HNO_3 in 15 mL metal-free polypropylene tubes and injected into an inductively-coupled plasma optical emission spectrometer (ICP-OES, Thermo Scientific icAP 7400, Waltham, MA, USA) to quantify all other elements in the *Serratia* ionome. ICP-OES analysis was validated and calibrated with aqueous multi-element external standard reference solutions (CPI International, Santa Rosa, CA, USA) and an in-line internal standard of Yttrium (CPI International, Santa Rosa, CA, USA) to correct for instrument drift or potential matrix effects. When the elemental concentrations were within the range of blank controls, they were considered to be at the detection limit of the instrument and excluded from further analysis. This resulted in 10 elements which were above LOD in all samples. We calculated nutrient use efficiencies (NUE) of each of these 10 elements and

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

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

$$ILR = SQRT\ (rs/r+s)\ \ln\ [g(c^{+})/g(c^{-})]$$ (Egozcue and Pawlowsky-Glahn 2005)

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

Results

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgments

PDJ thanks KP, TK, Mikko Mönkkönen, Jouni Taskinen and the Department of Biological and Environmental Science at the University of Jyväskylä for making this work possible. We thank Roghaieh Ashrafi for 16s PCR, Emmi Räsänen for lab assistance. Support from Fulbright Finland to PDJ, Academy of Finland grant #260704 to Jouni Taskinen and #278751 to TK, Finnish Cultural Foundation grant to KP, and US National Science Foundation grant #1930736 to PDJ is gratefully acknowledged. The authors thank the editor and anonymous reviewers at *RS Interface* for constructive comments.

References

Aachmann, F. L., M. Sørlie, G. Skjåk-Bræk, V. G. H. Eijsink, and G. Vaaje-Kolstad. 2012. NMR structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein dynamics, and substrate interactions. Proceedings of the National Academy of Sciences of the United States of America 109:18779–18784.

Acquisti, C., J. J. Elser, and S. Kumar. 2009. Ecological nitrogen limitation shapes the DNA composition of plant genomes. Molecular biology and evolution 26:953–956.

Aitchison, J. 1986. The statistical analysis of compositional data. Chapman and Hall, London; New York.

Angerer, A., B. Klupp, and V. Braun. 1992. Iron transport systems of Serratia marcescens. Journal of bacteriology 174:1378–1387.

Baudouin-Cornu, P., Y. Surdin-Kerjan, P. Marlière, and D. Thomas. 2001. Molecular evolution of protein atomic composition. Science 293:297–300.

Baxter, I. 2015. Should we treat the ionome as a combination of individual elements, or should we be deriving novel combined traits? Journal of experimental botany 66:2127–2131.

Baxter, I. R., O. Vitek, B. Lahner, B. Muthukumar, M. Borghi, J. Morrissey, M. L. Guerinot, et al. 2008. The leaf ionome as a multivariable system to detect a plant's physiological status. Proceedings of the National Academy of Sciences of the United States of America 105:12081–12086.

Bianchi, T. S. 2021. The evolution of biogeochemistry: revisited. Biogeochemistry:154: 141–181.

Björnerås, C., G. A. Weyhenmeyer, C. D. Evans, M. O. Gessner, H.-P. Grossart, K. Kangur, I. Kokorite, et al. 2017. Widespread Increases in Iron Concentration in European and North American Freshwaters: Increasing Iron Concentrations. Global biogeochemical cycles 31:1488–1500.

Browning, T. J., E. P. Achterberg, I. Rapp, A. Engel, E. M. Bertrand, A. Tagliabue, and C. M. Moore. 2017. Nutrient co-limitation at the boundary of an oceanic gyre. Nature 551:242–246.

Bruneaux, M., I. Kronholm, R. Ashrafi, and T. Ketola. 2022. Roles of adenine methylation and genetic mutations in adaptation to different temperatures in Serratia marcescens. Epigenetics: official journal of the DNA Methylation Society 17:861–881.

Casey, J. R., A. Mardinoglu, J. Nielsen, and D. M. Karl. 2016. Adaptive Evolution of Phosphorus Metabolism in Prochlorococcus. mSystems 1.

Droop, M. R. 1973. Some thoughts on nutrient limitation in algae. Journal of phycology 9:264–272.

Duetz, W. A., L. Ruedi, and R. Hermann. 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Applied and Environmental Microbiology 66:2641–2646.

Eide, D. J., S. Clark, T. M. Nair, M. Gehl, M. Gribskov, M. L. Guerinot, and J. F. Harper. 2005. Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in Saccharomyces cerevisiae. Genome biology 6:R77.

Egozcue, J. J., and V. Pawlowsky-Glahn. 2005. Groups of Parts and Their Balances in Compositional Data Analysis. Mathematical geology 37:795–828.

Elser, J. J., C. Acquisti, and S. Kumar. 2011. Stoichiogenomics: the evolutionary ecology of macromolecular elemental composition. Trends in ecology & evolution 26:38–44.

Elser, J., and P. Haygarth. 2020. Phosphorus: Past and Future. Oxford University Press.

Fitsanakis, V. A., N. Zhang, S. Garcia, and M. Aschner. 2010. Manganese (Mn) and iron (Fe): interdependency of transport and regulation. Neurotoxicity research 18:124–131.

Fay, P. A., S. M. Prober, W. S. Harpole, J. M. H. Knops, J. D. Bakker, E. T. Borer, E. M. Lind, et al. 2015. Grassland productivity limited by multiple nutrients. Nature plants 1:15080.

Friman, V.-P., T. Hiltunen, J. Laakso, and V. Kaitala. 2008. Availability of prey resources drives evolution of predator-prey interaction. Proceedings. Biological sciences / The Royal Society 275:1625–1633.

Flyg, C., K. Kenne, and H. G. Boman. 1980. Insect Pathogenic Properties of Serratia marcescens: Phage resistant Mutants with a Decreased Resistance to Cecropia Immunity and Dtxreased Virulence to Drosophila. Journal of general microbiology 120:173–181.

Fukushima, T., and B. Matsushita. 2021. Limiting nutrient and its use efficiency of phytoplankton in a shallow eutrophic lake, Lake Kasumigaura. Hydrobiologia 848:3469–3487.

Gounand, I., T. Daufresne, D. Gravel, C. Bouvier, T. Bouvier, M. Combe, C. Gougat-Barbera, et al. 2016. Size evolution in microorganisms masks trade-offs predicted by the growth rate hypothesis. Proceedings. Biological sciences / The Royal Society 283.

Grimont, P. A., and F. Grimont. 1978. The genus Serratia. Annual review of microbiology 32:221–248.

Harpole, W. S., J. T. Ngai, E. E. Cleland, E. W. Seabloom, E. T. Borer, M. E. S. Bracken, J. J. Elser, et al. 2011. Nutrient co-limitation of primary producer communities: Community co-limitation. Ecology letters 14:852–862.

Hofmann, P., A. Clark, P. Hoffmann, A. Chatzinotas, W. S. Harpole, and S. Dunker. 2021. Beyond nitrogen: phosphorus - estimating the minimum niche dimensionality for resource competition between phytoplankton. Ecology letters 24:761–771.

Huang, X.-Y., and D. E. Salt. 2016. Plant Ionomics: From Elemental Profiling to Environmental Adaptation. Molecular plant 9:787–797.

Isanta-Navarro, J., C. Prater, L. M. Peoples, I. Loladze, T. Phan, P. D. Jeyasingh, M. J. Church, et al. 2022. Revisiting the growth rate hypothesis: Towards a holistic stoichiometric understanding of growth. Ecology letters 25:2324–2339.

Jeyasingh, P. D., and L. J. Weider. 2007. Fundamental links between genes and elements: evolutionary implications of ecological stoichiometry. Molecular ecology 16:4649–4661.

Jeyasingh, P. D., J. M. Goos, P. R. Lind, P. Roy Chowdhury, and R. E. Sherman. 2020. Phosphorus supply shifts the quotas of multiple elements in algae and Daphnia: ionomic basis of stoichiometric constraints. Ecology letters 23:1064–1072.

Jeyasingh, P. D., J. M. Goos, S. K. Thompson, C. M. Godwin, and J. B. Cotner. 2017. Ecological Stoichiometry beyond Redfield: An Ionomic Perspective on Elemental Homeostasis. Frontiers in microbiology 8:722.

Jeyasingh, P. D., R. D. Cothran, and M. Tobler. 2014. Testing the ecological consequences of evolutionary change using elements. Ecology and evolution 4:528–538.

Kaim, W., B. Schwederski, and A. Klein. 2013. Bioinorganic Chemistry -- Inorganic Elements in the Chemistry of Life: An Introduction and Guide (2nd ed.). Wiley.

Kay, A. D., I. W. Ashton, E. Gorokhova, A. J. Kerkhoff, A. Liess, and E. Litchman. 2005. Toward a stoichiometric framework for evolutionary biology. Oikos 109:6–17.

Ketola, T., J. Laakso, V. Kaitala, and S. Airaksinen. 2004. Evolution of Hsp90 expression in *Tetrahymena thermophila* (Protozoa, Ciliata) populations exposed to thermally variable environments. Evolution; international journal of organic evolution 58:741–748.

Ketola, T., L. Mikonranta, and J. Mappes. 2016. Evolution of bacterial life-history traits is sensitive to community structure. Evolution 70:1334–1341.

Ketola, T., L. Mikonranta, J. Zhang, K. Saarinen, A.-M. Ormälä, V.-P. Friman, J. Mappes, et al. 2013. Fluctuating temperature leads to evolution of thermal generalism and preadaptation to novel environments. Evolution; international journal of organic evolution 67:2936–2944.

Kuo, P.-A., C.-H. Kuo, Y.-K. Lai, P. L. Graumann, and J. Tu. 2013. Phosphate limitation induces the intergeneric inhibition of Pseudomonas aeruginosa by Serratia marcescens isolated from paper machines. FEMS microbiology ecology 84:577–587.

Leal, M. C., O. Seehausen, and B. Matthews. 2017. The Ecology and Evolution of Stoichiometric Phenotypes. Trends in ecology & evolution 32:108–117.

Lenski, R. E. 2010. Phenotypic and Genomic Evolution during a 20,000-Generation Experiment with the bacterium *Escherichia coli*. Pages 225–265 *in* Plant Breeding Reviews. John Wiley & Sons, Inc., Oxford, UK.

Loladze, I. 2014. Hidden shift of the ionome of plants exposed to elevated CO_2depletes minerals at the base of human nutrition. eLife 3:e02245.

Merchant, S. S., and J. D. Helmann. 2012. Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation. Advances in microbial physiology 60:91–210.

NCBI 1112. https://www.ncbi.nlm.nih.gov/genome/1112?genome_assembly_id=233157

Novak, M., T. Pfeiffer, R. E. Lenski, U. Sauer, and S. Bonhoeffer. 2006. Experimental tests for an evolutionary trade-off between growth rate and yield in E. coli. The American naturalist 168:242–251.

Palmer, L. D., and E. P. Skaar. 2016. Transition Metals and Virulence in Bacteria. Annual review of genetics 50:67–91.

Parent S.-É. 2020. Why we should use balances and machine learning to diagnose ionomes. Authorea Prepr. doi: 10.22541/au.157954751.17355951.

Peñuelas, J., M. Fernández-Martínez, P. Ciais, D. Jou, S. Piao, M. Obersteiner, S. Vicca, et al. 2019. The bioelements, the elementome, and the biogeochemical niche. Ecology e02652.

Penuelas, J., J. Sardans, and J. Terradas. 2022. Increasing divergence between human and biological elementomes. Trends in ecology & evolution. doi.org/10.1016/j.tree.2022.08.007

Pittman, J. R., L. C. Kline, and W. J. Kenyon. 2015. Carbon-Starvation Induces Cross-Resistance to Thermal, Acid, and Oxidative Stress in Serratia marcescens. Microorganisms 3:746–758.

Poole, K., and V. Braun. 1988. Iron regulation of Serratia marcescens hemolysin gene expression. Infection and immunity 56:2967–2971.

Quigg, A., Z. V. Finkel, A. J. Irwin, Y. Rosenthal, T.-Y. Ho, J. R. Reinfelder, O. Schofield, et al. 2003. The evolutionary inheritance of elemental stoichiometry in marine phytoplankton. Nature 425:291–294.

Redfield, A. C. 1958. The biological control of chemical factors in the environment. American scientist 46:230A–221.

Rudman, S. M., J. M. Goos, J. B. Burant, K. V. Brix, T. C. Gibbons, C. J. Brauner, and P. D. Jeyasingh. 2019. Ionome and elemental transport kinetics shaped by parallel evolution in threespine stickleback. Ecology letters 22:645–653.

Rudman, S. M., M. A. Barbour, K. Csilléry, P. Gienapp, F. Guillaume, N. G. Hairston Jr, A. P. Hendry, et al. 2018. What genomic data can reveal about eco-evolutionary dynamics. Nature ecology & evolution 2:9–15.

Salt, D. E., I. Baxter, and B. Lahner. 2008. Ionomics and the study of the plant ionome. Annual review of plant biology 59:709–733.

San Roman, M., and A. Wagner. 2018. An enormous potential for niche construction through bacterial cross-feeding in a homogeneous environment. PLoS computational biology 14:e1006340.

Sardans, J., H. Vallicrosa, P. Zuccarini, G. Farré-Armengol, M. Fernández-Martínez, G. Peguero, A. Gargallo-Garriga, et al. 2021. Empirical support for the biogeochemical niche hypothesis in forest trees. Nature ecology & evolution.

Schindler, D. W. 1977. Evolution of phosphorus limitation in lakes. Science 195:260–262.

Schlesinger, W. H. 1997. Biogeochemistry: An Analysis of Global Change. Gulf Professional Publishing.

Sherman, R. E., R. Hartnett, E. L. Kiehnau, L. J. Weider, and P. D. Jeyasingh. 2021. Quantitative genetics of phosphorus content in the freshwater herbivore, Daphnia pulicaria. The Journal of animal ecology 90:909–916.

Sterner, R. W., and J. Elser. 2002. Ecological Stoichiometry. Princeton University Press, Princeton, N.J.

Turner, C. B., B. D. Wade, J. R. Meyer, B. A. Sommerfeld, and R. E. Lenski. 2017. Evolution of organismal stoichiometry in a long-term experiment with Escherichia coli. Royal Society open science 4:170497.

van der Ploeg, R. R., W. Bohm, and M. B. Kirkham. 1999. On the Origin of the Theory of Mineral Nutrition of Plants and the Law of the Minimum. Soil Science Society of America 63:1055.

Vitousek, P. 1982. Nutrient Cycling and Nutrient Use Efficiency. The American naturalist 119:553–572.

Warsi, O. M., and D. E. Dykhuizen. 2017. Evolutionary implications of Liebig's law of the minimum: Selection under low concentrations of two nonsubstitutable nutrients. Ecology and evolution 7:5296–5309.

Warsi, O. M., D. I. Andersson, and D. E. Dykhuizen. 2018. Different adaptive strategies in E. coli populations evolving under macronutrient limitation and metal ion limitation. BMC evolutionary biology 18:72.

Werner, A., and R. K. Kinne. 2001. Evolution of the Na-P(i) cotransport systems. American journal of physiology. Regulatory, integrative and comparative physiology 280:R301–12.

Williams, R. J. P., and J. J. R. F. da Silva. 2005. The Chemistry of Evolution: The Development of our Ecosystem (1 edition.). Elsevier Science.

Williams, R. J. P., R. Rickaby. 2012. Evolution's Destiny: Co-Evolving Chemistry of the Environment and Life. Royal Society of Chemistry.

Figures and Tables

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

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

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

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

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

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

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

(a) Function	Element	CV (%)
Building	C	4.8
	N	6.5
	P	21.0
	S	8.3
Balance	Ca	21.6
	K	35.1
	Mg	61.7
	Na	70.7
Catalysis	Fe	101.8
	Mn	129.4

(b) Lineage	CV (%)
Full A	13.8
Full D	73.9
LowC A	22.1
LowC D	29.6
LowN A	21.6
LowN D	77.9
LowP A	10.8
LowP D	24.1
LowFe A	15.2
LowFe D	11.7
LowMn A	17.0
LowMn D	21.7