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Rock geochemistry induces stress and starvation responses in the bacterial proteome

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Running title: Rock-driven changes in the bacterial proteome

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Summary

Interactions between microorganisms and rocks play an important role in Earth system processes. However, little is known about the molecular capabilities microorganisms require to live in rocky environments. Using a quantitative label-free proteomics approach, we show that a model bacterium (*Cupriavidus metallidurans* CH34) can use volcanic rock to satisfy some elemental requirements, resulting in increased rates of cell division in both magnesium- and iron-limited media. However, the rocks also introduced multiple new stresses via chemical changes associated with pH, elemental leaching and surface adsorption of nutrients that were reflected in the proteome. For example, the loss of bioavailable phosphorus was observed and resulted in the up-regulation of diverse phosphate limitation proteins which facilitate increase phosphate uptake and scavenging within the cell. Our results revealed that despite the provision of essential elements, rock chemistry drives complex metabolic reorganisation within rock-dwelling organisms, requiring tight regulation of cellular processes at the protein level. This study advances our ability to identify key microbial responses that enable life to persist in rock environments.

Introduction

The majority of Earth's biomass is located in sub-surface habitats. Indeed, Whitman *et al* (1998) suggest that the terrestrial subsurface has the largest reservoir of prokaryotic cells on Earth, whilst Kallmeyer *et al* (2012) estimate sub-seafloor prokaryotic abundance to be at least equal to that of seawater. These sub-surface habitats range from rocks just millimetres below the surface (Friedmann and Ocampo, 1976) to the deep continental and ocean crust (Mason *et al*., 2009; Reith, 2011; Orcutt *et al*., 2011; Nyyssönen *et al*., 2014).

In any sub-surface habitat, microorganisms will be in proximity to rocks, which are highly heterogeneous and reactive substrates (Jones and Bennett, 2013). Several studies have described the microbial release of bioessential elements from rocks (e.g Vandevivre *et al*., 1995; Uroz *et al*., 2009) and microbially induced changes in rock redox chemistry (e.g. Gadd, 2010 and references therein). However, even in the absence of active microbial weathering, rock chemistry can affect the structure, composition and metabolic activities of microbial communities (Mason *et al*., 2009; Kelly *et al*., 2011; Nyyssönen *et al*., 2014). Although these effects are well documented, these studies have relied on genome-level approaches that fail to capture the intracellular responses induced by environmental changes. This is particularly key in ecosystems with complex geochemistry, such as rock environments. Even transcriptomics-based approaches, which provide information on functional gene expression, are known to poorly correlate with protein expression (Nie *et al*., 2007). Proteomics technologies, however, enable quantification of thousands of proteins in a complex sample. This allows us to directly observe changes in the functions of cells in response to environmental perturbations. The large number of proteins detected makes

proteomics an excellent tool for capturing the complexity of bacterial responses to environmental changes (Armengaud, 2013).

We hypothesised that changes associated with the release of elements from rock, whilst potentially providing nutrients, would also subject cells to physiological stresses that are directly related to rock geochemistry. We explore this by quantifying changes in protein expression in the presence of rock using the bacterium *Cupriavidus metallidurans* CH34 as a model strain. This provides a snapshot of major processes occurring within the cells and allows us to capture the complexity of such an interaction. We conducted three major experiments: 1) We compare growth, chemical changes and protein expression with and without basaltic rock added to optimal growth media to explore the effects of the presence of rock on the bacterial proteome, 2) We then investigate the effect of increased pH caused by the addition of rock to disentangle the contribution of this perturbation to changes in the proteome, and 3) Finally we compare growth, chemical changes and protein expression with basalt added to nutrient-limited growth medium, to assess the ability of *C. metallidurans* CH34 to use basalt as a source of iron and magnesium.

Our results show that the presence of the rocks induced geochemical changes that correlate with changes in the proteome expression profiles of *C. metallidurans*. The shifts in protein expression are primarily associated with nutrient limitation and stress responses, despite the provision of some bioessential elements by the rock. These experiments provide new insights into the stressors and benefits for microorganisms dwelling in rocky environments.

Results

Changes in fluid chemistry and cell division

In order to assess how the presence of rock influences fluid chemistry and microbial processes, cells were cultured in 50 ml of "optimal growth media" (See Experimental Procedures) with 5 g of sterile basalt and fluid chemistry was measured. This was compared to abiotic changes in fluid geochemistry induced by the presence of rocks in identical conditions, but without cells. Addition of rock to optimal growth media (see Experimental Procedures) was associated with several geochemical changes in both *Cupriavidus*-inoculated (biotic) and non-inoculated (abiotic) experiments. At the end of the experiment, after 260 hours, phosphorus concentrations in the abiotic media had decreased in the presence of rock compared to media without rock ($t(2) = 73.2$, $P = 0.0002$, Fig. 1D). All abiotic conditions with rock resulted in significantly increased iron, calcium, silicon and aluminium concentrations in relation to control samples without rock (Fig. 1A, 1B, 1E, 1F; Fe $t(2) = 47.4$, $P = 0.0004$; Ca $t(2) = 42.6$, $P = 0.0006$; Si $t(2) = 89$, $P = 0.0001$; Al $t(2) = 70.6$, $P = 0.0001$). *Cupriavidus*-inoculated cultures had approximately the same concentration of phosphorus with rock as cultures without rock, both of which were much lower than in the original medium (Fig. 1D). Zinc and manganese concentrations in the supernatant of experiments with rock were consistently lower compared to media without rock (Fig. 1G, 1H; Mn $t(2) = 11.9$, $P = 0.007$; Zn $t(2) = 21.8$, $P = 0.002$).

In cultures without rock added, the pH remained stable at pH 7 in abiotic treatments but increased from pH 7 to approximately pH 7.5 in biotic experiments. In the presence of rock, in both *Cupriavidus*-inoculated and non-inoculated media, we observed an increase from pH 7 to approximately pH 8 (not shown). Cultures grown in optimal media in the presence of rock showed lower growth rates and lower final cell densities than cultures grown in optimal media (pH 7) without rocks (Fig. 2). To assess whether the reduction in cell density

in the presence of the rocks was caused by pH, cells were cultured in the same growth media, but with the pH increased from pH 7 to pH 8 and with no rock added. This led to a similar decrease in optical density as was observed in the presence of rock (Fig. 2).

Phosphorus partitioning assays were conducted to gain a detailed understanding of the changes in phosphorus chemistry in the experiments. In both the abiotic ($t(3) = 18.2314$, $P = <0.001$; Fig. 3A) and biotic ($t(3) = 6.2519$, $P = <0.001$; Fig. 3A) experiments, total phosphorus was observed to decrease in the presence of rock (Fig. 3). These data can be further analysed in relation to the different components of the phosphorus pool. In the biotic experiments most of the measured phosphorus was observed as particulate phosphorus, which is presumed to be cell biomass (this observation explains why ICP-OES did not detect a difference in phosphorus concentrations between biotic experiments with and without rock, since the filtering step in the methodology would remove particulate phosphorus). In the abiotic experiments, the phosphorus is primarily reactive soluble phosphorus (Fig. 3D). The lower phosphorus concentrations in the presence of rock in both abiotic and biotic experiments cannot be explained by precipitation of the phosphorus. Although theoretical calculations of the abiotic solution chemistry in the presence of rock show the solution is saturated with respect to three calcium phosphate minerals (See Supplementary Information, Fig. S1), the particulate phosphorus concentrations in the presence and absence of rock in the abiotic controls were negligible and no significant difference was observed ($t(1) = 2.094$, $P = 0.28$; Fig. 3C). The lower concentrations of soluble phosphorus in the presence of rock suggest removal of phosphorus from solution, perhaps by binding to the rock surface.

Proteome changes in the presence of basalt

A total of 1685 proteins were identified and quantified across all experimental treatments. This represents nearly 25 % of all of the protein-coding genes in the *C. metallidurans* CH34 genome (Janssen *et al.*, 2010). Good technical reproducibility across biological triplicates was observed, with each triplicate having a correlation coefficient of >0.99 when compared to the mean of the triplicates.

When the proteomes of cultures grown in optimal medium with and without rock were compared, fifty-two proteins have significantly higher abundance when rocks are present (3% of the detected proteins). Table 1 lists the proteins that were of higher abundance in cells grown in the presence of rock compared to cells grown in optimal media without rock. These included a diverse suite of proteins associated with low levels of phosphorus (Table 1). Putatively up-regulated proteins were associated with two phosphate limitation-related strategies: increase of phosphate uptake from outside of the cell and scavenging of phosphate from intracellular reserves (Table 1).

Proteins involved in import of phosphate into the cell from outside, represented pathways for transport of phosphate in three forms: phosphate, phosphonate and phosphite (see Table 1). These included the PstS protein from the phosphate-specific transport system that is involved in free phosphate import, and the high affinity phosphate uptake system proteins PhnD and PtxB that are likely involved in transport of phosphonate and phosphonate esters, or phosphite.

Proteins associated with intracellular phosphorus scavenging typically degrade larger phosphate-containing compounds. The patatin (PatD) and phospholipase C (PlcN) enzymes, for example, release phosphorus from phospholipids (Titball, 1993; Banerji and Flieger,

2004). PhoD is an alkaline phosphatase enzyme thought to be a scavenging mechanism by which bacteria generate free phosphate groups from many types of molecules.

In addition to the low phosphorus response, we observed an increased abundance of proteins involved in alternative energy generating pathways in the membrane and cellular redox homeostasis (Table 1). These included proteins associated with hydrogen oxidation (i.e. hydrogenotrophy) (oxygen-tolerant membrane-bound hydrogenase formation protein HoxQ) and an enzyme involved in sulphite oxidation (sulfite:cytochrome c oxidoreductase SorA). We also observed an increase in the abundance of the entire set of proteins involved in formate oxidation (FdhA, FdhB, Rmet_2759, FdhC). These Fdh enzymes catalyse the oxidation of formate to CO_2 and H^+ and have been associated with stress responses in *Desulfovibrio vulgaris* (Zhou *et al*., 2011).

The remaining proteins that have higher relative abundance in the conditions with rock present were primarily associated with diverse membrane and periplasmic transport processes, and regulatory processes such as signalling and transcription regulation (Table 1).

Forty-five proteins are significantly less abundant in the presence of rock relative to the control and are shown in Table 2. These represent 2.7% of the detected proteins.

We observed a decrease in abundance of metal cation responses and efflux systems with rock present. ZniA, ZniB and ZntA all have a specific affinity for zinc and cadmium. We also observed reduced relative abundance of the HmzP two-component transcriptional regulator, a metal cation resistance protein, and a ferric reductase (Rmet_3017) located downstream of *hmzP* in the genome.

Lower abundance of proteins associated with consumption of high energy, phosphorus-containing compounds such as ATP and NADPH was observed, relative to the control. For example, four ATPases have decreased abundance (Rmet_0297, Rmet_1426, Rmet_2164 and Rmet_3358), as are oxidoreductase enzymes associated with energy metabolism.

Other putatively down-regulated proteins included those involved in transcription, biotin synthesis, oxidoreductase reactions, transport and signalling (Table 2). Although typically associated with phosphorus homeostasis, an alkaline phosphatase and a phosphoesterase enzyme have decreased relative abundance in the presence of rock. However, these also had significantly lower abundance in the proteome of cells grown at pH 8 compared to the control (See following section). All proteins which have lower abundance both in the presence of rock and in cells cultured at pH 8 are highlighted in Table 2.

Proteome changes at pH 8

Across all experimental treatments, rock-induced variation in protein expression was accompanied by an increase in pH in the growth media. The effect of an increase in pH was characterised in isolation in order to separate the influence of pH from other factors introduced by the presence of rock. Final optical density at pH 8 (Fig. 2) was low in comparison with cells at pH 7. Growth rate and final cell density at pH 8 was indistinguishable from those observed in the presence of rock (Fig. 2).

Cultures grown in medium at pH 8 had 21 proteins with significantly higher abundance and 25 proteins with significantly lower abundance compared with cells grown in medium at pH 7. Only 5 of these were also of decreased abundance in the proteome of cells grown in rock-amended media and are highlighted in Table 2. In both cultures grown at pH 8 and those

grown in the presence of rock, a putative type-4 fimbrial biogenesis protein (PilY1), isocitrate lyase (AceA), alkaline phosphatase (PhoA1), acid phosphatase (AcpA) and a putative metallo-dependent amidohydrolase were significantly decreased in abundance. These proteins did not appear to be functionally related to one another and represented individual proteins, the production of which appeared to be pH-dependent.

Effect of initial element limitation on the proteome

To assess the importance of the rocks as providers of bioessential elements, experiments were conducted with basalt added to growth media deprived of iron or magnesium. A greater than two-fold decrease in mean cell density relative to the control was observed in medium without iron, and no growth was observed in medium without magnesium in the absence of rock (Fig. 4). The addition of rock to all of these media resulted in almost identical growth curves across all conditions, with these data being indistinguishable from the growth curves obtained for optimal medium with rock (Fig. 4).

Multivariate analysis of the protein expression data was performed for cultures incubated in optimal medium in the absence of rock, and three types of growth media (optimal growth medium, medium with iron removed and medium with magnesium removed) in the presence of rock (see Experimental Procedures). This identified two main clusters defined by the presence or absence of rocks (Fig. 5). While these clusters were only 20 % dissimilar (based on a group-average clustering of Bray-Curtis similarity values), the observed difference was highly significant (2-way nested PERMANOVA: pseudo-$F_{1,10}$ = 19.552, P = <0.001). A significant effect of medium type was also observed (2-way nested PERMANOVA: pseudo-$F_{3,10}$ = 4.060, P = 0.002). No significant variation in multivariate dispersion was

observed between samples incubated in the presence or absence of rock (PERMDISP: $F_{4,10}$ = 0.440, P = 0.916), or in different types of media (PERMDISP: $F_{1,10}$ = < 0.001, P = 0.983).

All of the three media types in the presence of rock were 85 % similar (Fig. 5). However, cultures with rock, but in which the medium was limited by magnesium or iron were more similar to one another than to cultures in optimal media in the presence of rock (Monte Carlo P for: optimal + rock vs no Fe + rock: 0.0327; optimal + rock vs no Mg + rock: 0.0228; no Mg + rock vs no Fe + rock: 0.209). Further information on these results is provided in the Supplementary Information (Table S3).

In the absence of rock, cultures at pH 8 showed a higher similarity to cultures at pH 7 than they did to any of the cultures in the presence of rock (Fig. 5) (Table S4).

Figure 6 shows that a core set of proteins were consistently increased or decreased (26 and 20 proteins, respectively) in the presence of rock, regardless of whether the cultures were starved of a specific element (Fe or Mg) or not. This observation is consistent with the results shown in Fig. 5, supporting our finding that differences in protein expression were primarily attributable to the presence or absence of rock, as opposed to elemental starvation.

Proteins differentially expressed in all conditions (optimal + rock, no iron + rock, and no magnesium + rock) are highlighted in Tables 1 and 2. The increased abundance of phosphate limitation proteins, the formate dehydrogenase regulon and proteins also differentially regulated at pH 8 (See Proteome Changes at pH 8) dominated this group of common proteins.

Discussion

To better understand the mechanisms microorganisms require to inhabit a rock environment, we investigated whether volcanic rocks induce specific microbial responses that are directly linked to geochemical changes induced by elemental leaching from rock. We used quantitative label-free proteomics to explore the molecular adaptations used by a model microorganism (*Cupriavidus metallidurans* CH34) grown in the presence of basalt.

Our results showed that when iron and magnesium was limited in the original medium, the addition of basalt was able to improve microbial growth, showing that basalt provides a source of these essential elements to the organisms. Nevertheless, the presence of basalt suppressed overall growth rates by increasing the pH from 7.0 to 8.0, outside the optimum growth pH for this organism.

An increase in pH in the presence of basalt is consistent with current knowledge on basalt glass dissolution, which begins with the release of monovalent and divalent cations (as observed in our elemental analysis) via metal-proton exchange that consumes protons and could increase fluid pH (Oelkers and Gislason, 2001). A decrease in both growth rate and final biomass abundance in the presence of rock, driven by abiotic rock-water interactions, shows how the rock itself can induce stress to organisms.

Whilst rock-induced shifts in pH appeared to drive the decrease in growth rate, multivariate analysis of the proteome data (Fig. 5) revealed that increased pH is not the main factor influencing protein expression in the presence of rock.

A common response observed across all conditions with rock present was the up-regulation of proteins associated with phosphate limitation (Table 1). Phosphorus is partitioned differently in biotic and abiotic treatments such that biotic treatments have high particulate

phosphorus concentrations (as phosphorus is partitioned into cells) and abiotic treatments have the highest concentration of soluble phosphorus. However, regardless of partitioning effects, the *total* phosphorus concentration is lower in the presence of rock both with and without cells. As three calcium phosphate minerals are super-saturated at the ionic concentrations observed, the loss of phosphorus could, theoretically, have been driven by calcium phosphate precipitation (Fig. S1). However, the lack of particulate phosphate in experiments with rock rules out abiotic precipitate formation (Fig. 3C). Particulate phosphorus is high in biotic cultures and soluble (total and reactive) phosphorus is low but this is a result of soluble phosphorus uptake by cells and not mineral precipitation. Together, the low abiotic precipitation and low total phosphorus concentrations when rock is added, are consistent with phosphate sorption onto the rock surface rather than phosphate mineral formation. The sorption of phosphates to mineral surfaces has been studied extensively and occurs when phosphates sorb to metal oxyhydroxides via ligand exchange, an OH^- or an H_2O molecule is released from the surface resulting in the formation of a phosphate surface complex (e.g. Frossard *et al*., 1995). It is likely that this phosphorus sequestration results in exhaustion of available P before all the carbon source has been used and drives cells into an early, phosphorus limitation-induced stationary phase in the presence of rock as well as acting to limit biomass yield and drive protein expression.

These results show that the presence of rock not only induce stress by changing the pH of the solution, but also by reducing phosphorus availability, exerting a dominant effect on microbial proteome response. The observation that the proteome response was 85% similar regardless of whether the original medium was complete or limited in iron or magnesium shows that in rock environments, the stress induced by the mere presence of rock has the

potential to be a more prominent influence on microbial physiology than micronutrient limitation.

It is unclear whether *C. metallidurans* CH34 has the ability to solubilise mineral phosphates, facilitating the direct scavenging of phosphorus from a rock surface, or whether the increased abundance of these proteins assists in the accumulation of phosphate from organic sources. However, the ability of *C. metallidurans* to overcome phosphorus sequestration could confer a key advantage in the colonisation of rock habitats which are characterised by numerous pathways for phosphate removal.

We also observed an up-regulation of proteins involved in alternative metabolisms such as hydrogen and sulphite oxidation (Table 1) in the presence of rock. This may result from a need to utilise a diverse range of energy-producing processes because of the lack of phosphorus. In particular, the up-regulation of the formate dehydrogenase operon (observed here in all conditions in the presence of rocks) has been linked to stress responses by previous studies. For example, in *Desulfovibrio vulgaris,* the expression of the *fdhBAC* genes is associated with diverse stress responses where they are thought to be involved in enhancing energy required for stress-alleviating processes by increasing the flow of reducing equivalents through formate (Zhou *et al*., 2011). Consistent with this is the down-regulation of proteins associated with high energy phosphorus-containing compounds such as ATP (Table 2) that probably act to limit the consumption of these molecules due to their low availability.

Consistent with the reduction of zinc and manganese concentrations in the presence of rock, we observed a decreased abundance of various zinc efflux proteins. It is unclear why the concentrations of these cations are lower in the presence of rock, but nevertheless, the

304 results suggest a direct response of the bacterial proteome to the presence of trace
305 elements.

306 From both the element and proteome analysis, there is no evidence that *C. metallidurans*
307 CH34 has an active role in rock weathering in this case. Our observations are more
308 consistent with passive uptake of abiotically leached elements, and cellular responses to
309 abiotic surface reactions induced by the fluid-rock interactions.

310 By modifying the pH, releasing elements and sequestering phosphorus, rocks impose upon
311 cells a multiple-stress extreme environment that influences cell growth and requires the up
312 and down-regulation of a diverse suite of proteins. The use of label-free quantitative
313 proteomics has helped us capture the diversity of this response, highlighting the complex
314 array of physiological changes that microorganisms elicit in response to rock environments.
315 A comprehensive understanding of geochemistry must be coupled with detailed knowledge
316 of microbial responses to nutrient starvation and other physiological stresses, if we are to
317 truly understand the factors that drive the ability of microorganisms to colonise and actively
318 persist within rock environments. More broadly, this study highlights the need to quantify
319 the subtle complexities of microbial interactions with their environment.

320 **Experimental Procedures**

321 ***Substrate and organism selection***

322 The Gram-negative beta-proteobacterium *Cupriavidus metallidurans* CH34 was the model
323 organism for this study. *C. metallidurans* CH34 was selected as it has a versatile suite of
324 metabolic capabilities, is found within numerous natural and anthropogenic extreme
325 environments, and is well suited to life in stressful rock habitats (Olsson-Francis *et al.*, 2010).

It is particularly well-known for its resistance to heavy metals (Janssen *et al.*, 2010; Monsieurs *et al*., 2011). In addition, the full genome sequence of this strain is available and its gene functions are well-annotated, making *C. metallidurans* an excellent model organism for proteomics studies (Janssen *et al*., 2010).

We used a poorly crystalline basaltic rock as the model substrate. Basalt is a common igneous rock that, due to the active volcanism present early in Earth history, is likely to have been very important for early life (Moorbath, 2005). In a poorly crystalline rock, elements are more homogeneously distributed throughout the substrate rather than partitioned into specific minerals. As each experimental replicate contains a sub-sample of rock, this helps make our experiments more controlled and reproducible by reducing the variability between rocks used in each flask.

The basalt was collected from Skaptafell, Iceland (64°45'58"N 23°38'59"W). The rock composition, as determined by X-Ray Fluorescence (PANalytical PW2404, PANalytical, UK), was 44% SiO_2, 15% Al_2O_3, 12% CaO, 11% Fe_2O_3, 7% MgO, 3% TiO_2, 1% Na_2O, 1% K_2O, 0.4% P_2O_5, 0.2% MnO, with 5.6% lost on ignition. The rock also contained trace elements in the following concentrations: 426ppm Sr, 353ppm V, 303ppm Cr, 284ppm Ba, 139ppm Cu, 128ppm Zr, 111ppm Zn, 98ppm Ni, 45ppm Sc, 55ppm Ce, 36ppm Nb, 29ppm Nd, 22ppm La, 19ppm Y, 17ppm Rb, 1.5ppm Th, and 1.2ppm Pb. The basalt was crushed and sieved to isolate the 1 - 2.5 mm size fraction. This was rinsed in ddH_2O and dried overnight at room temperature before autoclaving at 121°C for 20 minutes.

Culturing and growth

348 *C. metallidurans* was routinely cultured at 30°C in Tris salts minimal medium (MM284) at pH
349 7 with 0.2 % (w/v) sodium gluconate and ferric ammonium citrate as the iron source, as
350 described previously (Mergeay *et al.,* 1985).

351 Reagent grade chemicals were used for media preparation (Sigma-Aldrich, UK). Previously
352 described MM284 has only enough phosphorus (as $Na_2HPO_4.2H_2O$) for complete
353 consumption of the carbon source. The onset of the stationary phase is triggered when the
354 carbon source is exhausted but phosphorus is also low by this time point. We do not
355 supplement the medium with an excess of additional phosphorus.

356 In order to assess stresses induced by the presence of rock, we cultured cells (starting cell
357 concentration = 2 x 10^4 CFU ml^{-1}) in 50 ml of "optimal growth media" (i.e. MM284 +
358 gluconate) with 5 g of sterile basalt added. All cultures were grown in acid-washed (to
359 remove any metal contamination), sterile 100 ml Nalgene polymethylpentene flasks instead
360 of glass flasks that occasionally interfere with inorganic leaching analysis. Cultures were
361 capped with sterile foam bungs to allow gas exchange with the atmosphere. Each test
362 condition was run in triplicate (n = 3). As it was unclear whether the presence of rock would
363 subject the cells to physical shear stress during continuous shaking, experiments were
364 conducted under static conditions with manual mixing at 24-hour intervals. Optical density
365 at 600 nm was measured daily by visible spectrometry (FLUOstar Optima, BMG Labtech, UK)
366 as a proxy for cell growth. Optical density was measured on 200µl of culture in a 96 well
367 microplate (path length = 6.31mm).

368 A complete set of abiotic controls were run in tandem by adding 50 ml of media to 5 g
369 sterile basalt in triplicate. These were not inoculated with cells and were kept at 30°C
370 alongside the biotic replicates and also shaken every 24 hours. The control samples were

used to quantify abiotic leaching from the rocks and to ensure that no particulate material might interfere with optical density measurements in the cultures with cells.

To isolate effects related to pH changes we also investigated the proteomes of cells grown in the same media (i.e. MM284 + gluconate) with the pH adjusted to pH 8 (with NaOH). To compare the proteomes of cells initially limited of a specific element, and thus required to obtain that element from the rock, we used media with the (Fe(III)NH_4.citrate) or magnesium ($MgCl_2$. $6H_2O$) source omitted and basalt added. These elements were selected as they comprise two of the main inorganic cations required for bacterial growth and are major constituents of basaltic rocks (Madigan *et al*., 2014). Optical density was also monitored in the iron- or magnesium-limited media alone to establish if the basalt was providing elements essential to growth. Abiotic triplicates of iron- and magnesium-limited media with rock were established as described above.

The experiment was conducted for 260 hours after which cells from all flasks were harvested and all following analyses conducted.

Elemental analysis

Final inorganic element concentrations at the end of the experiment were measured to compare changes in chemistry induced by the rocks and cells during the experiment. Cultures were centrifuged (4500 rpm, 5 minutes, 10°C), the supernatant filtered through a 0.22µm filter and final inorganic element concentrations in the cell-free supernatant determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES). Details on the analysis are provided in the Supplementary Information and in Table S1.

Phosphorus partitioning assays

To better understand phosphorus chemistry in the experiments, phosphorus partitioning assays were conducted to complement the ICP-OES analysis. The ICP-OES phosphorus analysis indicates only the phosphorus concentrations after the sample has been filtered and does not indicate what form of phosphorus is present. The phosphorus partitioning assays resolves the concentration of phosphorus that is: soluble and reactive, soluble and unreactive and in a particulate form. Particulate phosphorus is not measured directly but calculated by subtracting the concentration of the total soluble phosphorus (TSP) from the total phosphorus (TP).

Soluble Reactive Phosphorus (SRP) concentrations were determined following the method of Murphy & Riley (1962). This method uses a reagent of ammonium molybdate, potassium antimony tartrate, and L-ascorbic acid in 1 M of sulphuric acid, which reacts with the phosphate ion to form a phospho-molybdenum blue complex. Concentrations were determined by measuring absorbance at 882 nm in relation to known standards. Total phosphorus (TP) concentrations were determined on unfiltered samples, which were digested using a solution of sulphuric acid and potassium persulphate to convert all forms of phosphorus to SRP, which was then measured in a similar way to that described above. The method used was as described for TP by Wetzel and Likens (2000), with an added acidification step (0.1 ml of 30 % H_2SO_4 was added to the samples before addition of persulfate). Total Soluble Phosphorus (TSP) concentrations were determined in the same way as described for Total Phosphorus, but using a filtered sample. Particulate phosphorus concentrations were calculated by subtracting TSP from TP.

Proteome analysis

Cells were harvested by centrifugation (4500 rpm, 5 minutes, 10°C) after 256 hours of growth. Cells were lysed in 8M sterile urea with regular vortexing to release proteins. Proteins were broken down into their constituent peptides by trypsin digest (LeBihan *et al*., 2010). Peptides were analysed on a reverse phase microcolumn using a 140 minute gradient (controlled by a binary HPLC system 1200, Agilent, UK) coupled to a hybrid LTQ-Orbitrap XL mass spectrometer (Thermo-Fisher, UK) in data dependent mode, controlled through Xcalibur 2.0.7 software as described previously (Martin *et al*., 2012; Le Bihan *et al*., 2010). Eight microliters of sample in loading buffer was injected onto the analysis column.

Peak selection, normalisation and quantification were performed using Progenesis LC-MS (version 4.0, Nonlinear Dynamics, UK). Peptides (charges 2+, 3+ and 4+) were identified by MASCOT (Matrix sciences, UK, version 2.3) searches of MSMS data against the NCBI protein database subset (Tatusova *et al*., 2014) for *Cupriavidus metallidurans* (6766 sequences), using a trypsin/p enzyme restriction with a maximum missed-cut value of 2. Variable methionine oxidation and fixed cysteine carbamidomethylation were used in all searches. Precursor mass tolerance was set to 7 ppm and MSMS tolerance to 0.4 amu. The significance threshold (p) was set below 0.05 (MudPIT scoring).

P-values for fold changes between experimental condition and control (media without rock) were determined by one-way ANOVA on arcsinh-transformed protein intensities in Progenesis LC-MS (LeBihan *et al*., 2010). Differentially expressed proteins were considered significant with an average intensity ratio of at least two-fold and a *P*-value less than 0.05 if detected with two or more peptides per protein with a MASCOT identification score greater than 20.

Protein functions were assigned using functional annotation available on the UniProt Protein Knowledge Base (The UniProt Consortium, 2014) and cross-referenced with expert manual annotation of the Cupriavidu2Scope Project on the MAGE platform (Janssen *et al*., 2010).

A Bray-Curtis similarity matrix based on the protein expression data was analysed using the PRIMER statistical package (version 6.1.13) with the PERMANOVA+ add-on (version 1.0.3) (Clarke & Gorley, 2006; Anderson *et al*., 2008). Following non-metric multidimensional scaling ordination, a 2-way nested permutational analysis of variance (PERMANOVA; Anderson *et al*., 2001) was performed with 'Medium type' and 'Presence of rock' as the factors (Type III sums of squares, 9999 unrestricted permutations of the raw data). Since different sets of growth media were used in the presence and absence of rock, the factor 'Medium type' was nested under the factor 'Presence of rock'. Post-hoc pairwise comparisons were performed using the same PERMANOVA settings, with the exception of *P* values being derived by a Monte Carlo approach due to low numbers of permutations (Anderson *et al*., 2008). Variation in within-group dispersion was assessed using a test for the homogeneity of multivariate dispersions (PERMDISP, 9999 permutations) (Anderson, 2006).

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460 Supplementary information is available at the Environmental Microbiology website.

461 The authors have no conflict of interest.

462

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564

Figures

Figure 1. Comparison of final inorganic ion concentrations in culture supernatant of optimal medium condition and optimal media with rock added (biotic and abiotic) at the end of the experiment as analysed by ICP-OES. Abiotic refers to treatments not inoculated with cells, biotic refers to those which have been inoculated. Light grey = rock added, Dark grey = no rock. A) Iron concentration B) Calcium concentration C) Magnesium concentration D) Phosphorus concentration E) Silicon concentration F) Aluminium concentration G) Zinc concentration H) Manganese concentration. Abiotic = non-inoculated treatments, Biotic = inoculated treatments. Data shown are means ± SD (*n* = 2 except in biotic with rock, *n* = 3).

Figure 2. Growth curves of *Cupriavidus metallidurans* CH34 in optimal medium at pH 7, at pH 8 and with basaltic rock. The data shown are means ± SD (n=3).

Figure 3. Comparison of a) Total phosphorus b) Total Soluble Phosphorus c) Particulate Phosphorus and d) Soluble Reactive Phosphorus measured with and without rock present in inoculated and non-inoculated cultures. Data are shown as means ± SD (*n* = 3).

Figure 4. Comparison of growth of *Cupriavidus metallidurans* CH34 in different nutrient availability conditions showing improvement in growth compared to magnesium and iron starved cultures with all rock-containing conditions falling within a similar range regardless of initial nutrient conditions. Data are shown as means ± SD (*n* = 3). Labels: optimal pH7 = optimal media at pH 7 with no rock, optimal + rock = optimal media with rock present, - Fe +

rock = media with no iron added but rock present, - Fe no rock = media with no iron added and no rock present, - Mg no rock = media with no magnesium added and no rock present, - Mg + rock = media with no magnesium added but rock present. Abiotic refers to treatments not inoculated with cells, biotic refers to those which have been inoculated.

Figure 5. Non-metric multidimensional scaling (nMDS) ordination of *Cupriavidus metallidurans* CH34 cultures incubated in different types of growth media in the presence or absence of basalt. The ordination (stress = 0.07) was derived from a Bray-Curtis similarity matrix calculated from normalised protein expression data (Materials and Methods). Similarity thresholds (%) are based on group-average clustering. See Figure 4 for label descriptions.

Figure 6. Venn diagrams displaying the number of proteins which show similar increases or decreases in abundance in different experimental groups

Supplementary Information Figure Captions

Figure S1: Logarithmic mineral saturation index in non-inoculated flasks of optimal media plus rock shows that the fluid is super-saturated with respect to the phosphate minerals hydroxyapatite, vivianite and whittlockite. Temperature was fixed at 30°C and pH was fixed at pH 8.

606 **Table 1. Proteins with increased abundance in optimal media with rock added compared to optimal media without rock added.**

Replicon	Protein description	Protein name	Locus Tag	COG classes	Uniprot accession no.	X = common to all with rock	MASCOT score	No. peptides used for identification	Fold change	*p*-value
Phosphorus limitation										
CHROM 1	periplasmic phosphate binding protein	PstS	Rmet_2185	P	YP_584333.1	-	2864	36	2.15	1.8E-03
CHROM 1	phosphonate C-P lyase system	PhnL	Rmet_0761	P	YP_582916.1	x	27	5	2.71	7.8E-03
CHROM 1	phosphonate-binding periplasmic protein	PhnD	Rmet_0774	P	YP_582929.1	-	2128	32	2.02	5.5E-04
CHROM 1	phosphonate-binding periplasmic protein	PtxB	Rmet_2994	P	YP_585136.1	-	2675	27	2.03	6.8E-03
CHROM 1	putative Patatin-like phospholipase	PatD	Rmet_0151	R	YP_582306.1	x	152	3	2.11	2.4E-04
CHROM 2	phospholipase C	PlcN	Rmet_4192	M	YP_586328.1	x	1464	31	2.98	3.5E-03
CHROM 1	alkaline phosphatase	PhoD	Rmet_2583	P	YP_584729.1	x	298	9	5.84	1.7E-04
Alternative energy metabolisms										
CHROM 1	membrane-bound [NiFe]-hydrogenase formation protein, involved in nickel incorporation	HoxQ	Rmet_1290	-	YP_583444.1	x	132	4	2.44	7.9E-03
CHROM 2	sulfite:cytochrome c oxidoreductase molybdenum subunit	SorA	Rmet_4891	R	YP_587022.1	-	141	5	2.27	2.8E-03
CHROM 1	formate dehydrogenase, gamma subunit	FdhC	Rmet_2758	C	YP_584900.1	x	132	4	4.21	4.5E-03
CHROM 1	conserved hypothetical protein; putative exported lipoprotein	-	Rmet_2759	-	YP_584901.1	x	102	3	7.42	6.6E-05
CHROM 1	formate dehydrogenase, beta subunit with 4Fe-4S iron-sulfur domain	FdhB	Rmet_2760	C	YP_584902.1	x	299	7	10.82	6.5E-05
CHROM 1	formate dehydrogenase alpha subunit, with Fe4-S4 domain	FdhA	Rmet_2761	C	YP_584903.1	x	1109	24	4.15	1.1E-04
CHROM 1	disulfide oxidoreductase	-	Rmet_0109	Q	YP_582264.1	-	109	4	2.07	2.4E-03

CHROM 1	aldo/keto reductase	-	Rmet_3081	R	YP_585222.1	-	69	5	3.02	5.7E-03
Membrane & periplasm proteins										
CHROM 1	colicin transporter of the, tol-pal system, periplasmic component	TolB	Rmet_2675	U	YP_584817.1	-	799	15	2.05	5.0E-03
CHROM 1	taurine ABC-type transporter, periplasmic component	TauA	Rmet_2859	P	YP_585001.1	-	109	8	2.13	2.2E-02
CHROM 2	DL-methionine ABC-type transporter, periplasmic substrate-binding component	MetQ	Rmet_4988	P	YP_587119.1	-	526	8	2.47	6.9E-04
CHROM 2	ABC-type transporter involved in toluene tolerance, periplasmic component	Ttg2	Rmet_4167	Q	YP_586303.1	-	424	10	2.09	3.0E-05
CHROM 1	ABC-type transporter, solute-binding periplasmic component	-	Rmet_2122	P	YP_584270.1	-	265	10	2.29	3.8E-03
CHROM 2	ABC-type transporter, periplasmic component*	-	Rmet_5638	-	YP_587766.1	x	456	7	3.02	2.5E-04
CHROM 2	metal cation RND-type transporter, membrane protein	HmyB	Rmet_4121	M	YP_586257.1	x	404	8	7.85	5.0E-06
CHROM 1	putative lipoprotein	-	Rmet_3083	S	YP_585224.1	-	190	6	2.59	5.3E-03
CHROM 1	Putative lipoprotein precursor	-	Rmet_0997	-	YP_583152.1	x	33	3	4.59	1.2E-03
CHROM 1	beta N-acetyl-glucosaminidase	NagZ	Rmet_2413	G	YP_584559.1	x	163	6	5.70	5.1E-04
CHROM 1	phosphotransferase involved in extracellular matrix synthesis	EplL	Rmet_2726	M	YP_584868.1	-	47	2	2.38	4.9E-02
CHROM 1	surface antigen-like outer membrane lipoprotein	-	Rmet_0849	M	YP_583004.1	x	99	3	2.63	2.4E-04
CHROM 2	Extra-cytoplasmic solute receptor, periplasmic protein	Bug	Rmet_5294	S	YP_587422.1	x	50	5	4.79	2.5E-03
CHROM 2	Extra-cytoplasmic solute receptor, periplasmic protein	Bug	Rmet_5869	S	YP_587997.1	-	51	2	2.78	4.9E-04
CHROM 1	Extra-cytoplasmic solute receptor, periplasmic protein	Bug	Rmet_0982	S	YP_583137.1	-	199	7	2.30	9.4E-05
CHROM 1	Extra-cytoplasmic solute receptor, periplasmic protein	Bug	Rmet_1184	S	YP_583339.1	-	481	11	2.07	1.5E-03
Replication & Transcription & Translation										

CHROM 1	DNA polymerase III subunit	HolC	Rmet_2806	L	YP_584948.1	-	20	2	2.04	5.7E-04
CHROM 1	ribonucleotide-diphosphate reductase, subunit beta	NrdB	Rmet_3087	F	YP_585228.1	-	217	4	2.01	7.2E-03
CHROM 1	cell division protein	ZapD	Rmet_3112	S	YP_585253.1	-	214	5	2.46	1.8E-02
CHROM 1	LysR family transcriptional regulator		Rmet_3446	K	YP_585587.1	x	34	3	2.45	3.4E-04
CHROM 2	GntR family transcriptional regulator	-	Rmet_5631	K	YP_587759.1	x	28	5	9.01	2.5E-03
CHROM 1	BolA family transcriptional regulator	BolA	Rmet_3251	K	YP_585392.1	x	29	3	5.13	1.8E-03
CHROM 2	cold-shock responsive transcriptional repressor	Csp	Rmet_5818	K	YP_587946.1	-	257	3	2.39	1.0E-02
CHROM 1	50S ribosomal protein L28	RpmB	Rmet_2870	J	YP_585012.1	x	137	3	2.03	3.7E-02
Other										
CHROM 2	short-chain dehydrogenase/reductase SDR	FabG	Rmet_4414	IQR	YP_586548.1	-	39	3	2.50	8.5E-03
CHROM 1	methylmalonate-semialdehyde dehydrogenase	MmsA	Rmet_0206	C	YP_582361.1	-	361	9	2.01	1.5E-03
CHROM 1	acyl-CoA-binding protein	-	Rmet_1394	I	YP_583546.1	-	184	7	2.03	6.6E-04
CHROM 1	hemolysin-like Acyl-CoA N-acyltransferase	-	Rmet_2176	R	YP_584324.1	-	29	2	2.45	8.8E-03
CHROM 2	GCN5-related N-acetyltransferase	-	Rmet_5884	KR	YP_588012.1	x	58	4	4.26	2.4E-04
CHROM 2	homogentisate 1,2-dioxygenase, involved in phenylalanine & tyrosine degradation	HmgA	Rmet_4374	Q	YP_586508.1	x	54	2	4.66	4.5E-04
CHROM 2	putative glyoxalase or dioxygenase	-	Rmet_4030	E	YP_586167.1	x	71	2	3.13	1.0E-03
CHROM 1	conserved hypothetical protein	-	Rmet_2632	-	YP_584778.1	-	183	5	2.32	5.7E-03
CHROM 1	diguanylate cyclase	PleD	Rmet_0867	T	YP_583022.1	-	66	5	3.20	4.7E-02
CHROM 1	phosphoglycolate phosphatase	CbbZ1	Rmet_1514	R	YP_583666.1	x	44	2	3.46	6.7E-03
CHROM 1	putative polyphosphate kinase	Ppk	Rmet_0550	S	YP_582705.1	x	114	7	2.58	3.2E-02
CHROM 1	sulfate/thiosulfate import ATP-binding protein	CysA	Rmet_1378	P	YP_583530.1	-	62	2	2.58	3.4E-03

CHROM 1	sulphate adenylyltransferase subunit 1	CysN	Rmet_2812	P	YP_584954.1	x	852	14	2.84	8.8E-04

607

608 Differentially expressed proteins were considered biologically significant with an average intensity ratio of at least two-fold and a *p*-value less

609 than 0.05 if detected with two or more peptides per protein with a MASCOT identification score greater than 20. P(MC) = Monte Carlo *P*-value.

610 Column "X = common to all with rock" indicates whether this protein was also increased in abundance in the other media types with rock

611 added i.e. minus Fe + rock and minus Mg + rock.

612

613 **Table 2. Proteins with lower abundance in optimal media with rock added compared to optimal media without rock added.**

Replicon	Protein description	Protein Name	Gene Locus tag	COG classes	Uniprot accession no.	X = common to all rock conditions	MASCOT score	Peptides used for identification	Fold change	*p*-value
Metal homeostasis										
CHROM1	transcriptional regulator, part of two component system with HmzS	HmzR	Rmet_3016	TK	YP_585158.1	-	64	6	>500	2.8E-04
CHROM1	ferric reductase, FAD/NAD(P)-binding	-	Rmet_3017	P	YP_585159.1	x	71	4	15.1	2.6E-03
CHROM2	P-type ATPase involved in Zn(II), Cd(II), Ti(I) and Pb(II) resistance	ZntA	Rmet_4594	P	YP_586725.1	x	190	8	4.4	1.1E-02
CHROM2	RND metal efflux pump, part B	ZniB	Rmet_5320	M	YP_587448.1	x	198	7	2.3	8.6E-03
CHROM2	RND metal efflux pump, part A	ZniA	Rmet_5319	P	YP_587447.1	-	73	8	2.9	2.7E-02
Transport over membrane										
CHROM1	glycine betaine/carnitine/choline ABC-type transporter, periplasmic component	-	Rmet_0799	E	YP_582954.1	x	45	6	2.7	1.2E-02
CHROM1	ABC-type transporter, periplasmic component: HAAT family	-	Rmet_0920	E	YP_583075.1	-	67	5	2.3	3.1E-02
CHROM1	ABC-type transporter, periplasmic component: HAAT family	-	Rmet_2820	E	YP_584962.1	x	51	3	2.8	1.1E-02
CHROM1	ABC-type transporter subunit, ATP-binding	YadG	Rmet_3253	V	YP_585394.1	-	136	7	2.4	2.9E-03

	component									
CHROM1	ABC-type transporter, ATP-binding and membrane component	VcaM	Rmet_2516	V	YP_584662.1	x	87	5	7.1	2.0E-02
CHROM1	TRAP-type mannitol/chloroaromatic compound transporter, periplasmic component	-	Rmet_3543	Q	YP_585684.1	-	873	16	2.1	1.5E-04
CHROM1	putative oligoketide cyclase/lipid transport protein	YfjG	Rmet_1457	I	YP_583609.1	-	51	2	30.5	1.3E-02
CHROM1	import inner membrane translocase	-	Rmet_0372	S	YP_582527.1	x	491	9	2.2	1.1E-04
CHROM2	Extra-cytoplasmic solute receptor protein	Bug	Rmet_5038	S	YP_587169.1	-	23	3	3.3	1.5E-03
Transcription										
CHROM2	XRE family transcriptional regulator	-	Rmet_4373	K	YP_586507.1	-	47	2	2.3	2.6E-02
CHROM2	TetR family transcriptional regulator	-	Rmet_4909	K	YP_587040.1	x	48	4	4.5	3.6E-02
CHROM1	LysR family transcriptional regulator	YcaN	Rmet_1897	K	YP_584045.1	-	11	6	2.5	5.4E-03
CHROM1	transcription termination factor	Rho	Rmet_2135	K	YP_584283.1	x	499	13	2.7	7.9E-04
CHROM2	purine-binding chemotaxis regulator	CheW	Rmet_3681	NT	YP_585822.1	-	164	8	2.3	2.5E-03
pMOL28	histone-like bacterial DNA-binding protein	HupB	Rmet_6397	L	YP_003518271.1	-	263	6	4.2	1.8E-02
Biotin synthesis										
CHROM1	adenosylmethionine-8-amino-7-oxononanoate	BioA	Rmet_0114	H	YP_582269.1	x	389	8	2.8	1.2E-03

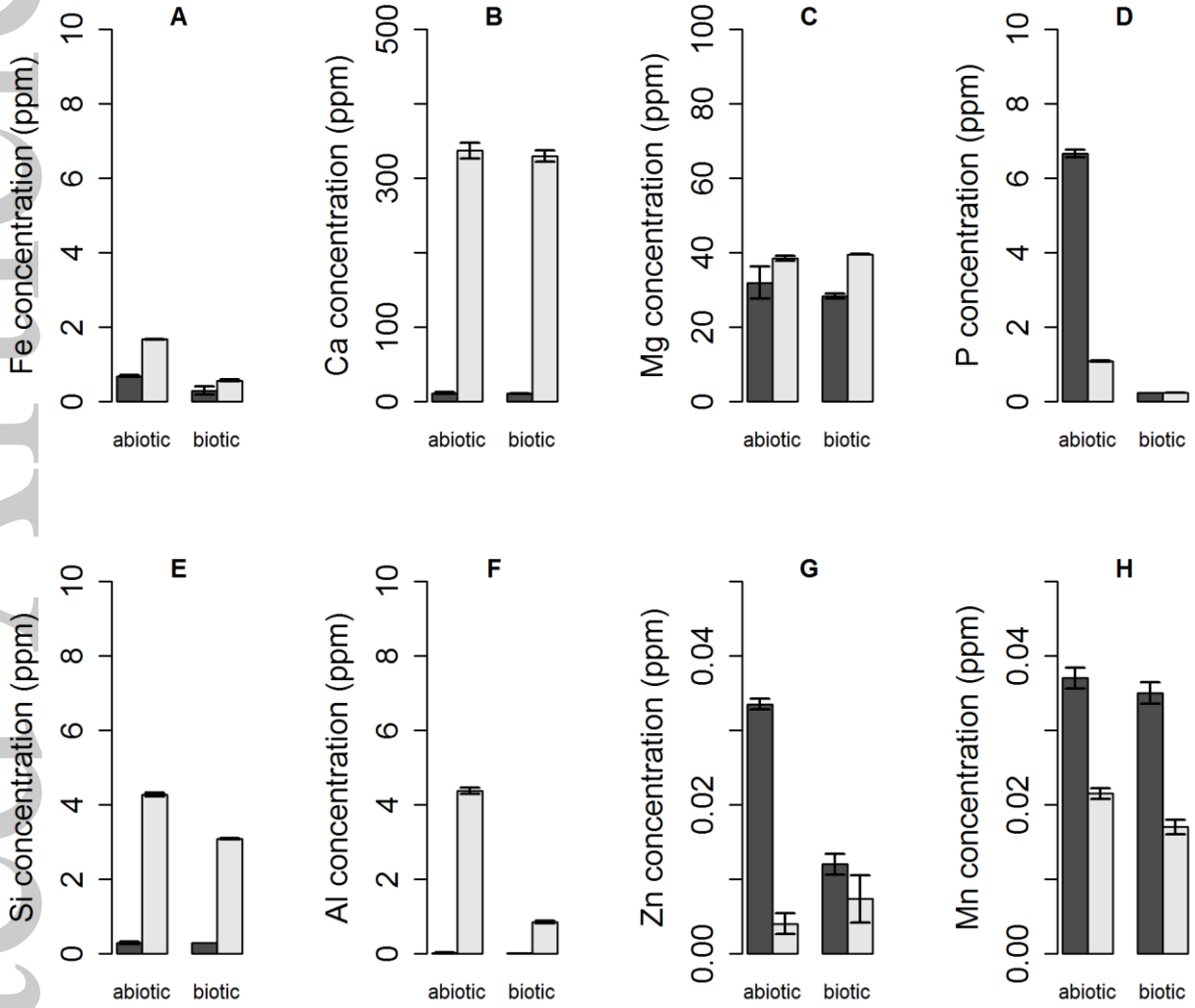
	transaminase									
CHROM1	amino-7-oxononanoate synthase	BioF	Rmet_0115	H	YP_582270.1	-	22	4	>600	1.3E-04
Oxidoreductases										
CHROM1	NADH dehydrogenase, subunit J	NuoJ	Rmet_0936	C	YP_583091.1	x	79	5	2.9	4.4E-03
CHROM1	thiosulphate-binding sulfur oxidation protein	SoxZ	Rmet_3422	-	YP_585563.1	-	35	2	2.5	1.3E-02
CHROM2	short-chain dehydrogenase/reductase SDR	-	Rmet_4614	IQR	YP_586745.1	x	79	6	2.4	2.2E-02
CHROM2	2-dehydropantoate 2-reductase	PanE	Rmet_5770	H	YP_587898.1	x	125	6	2.2	1.3E-03
CHROM1	NAD-dependent formate dehydrogenase alpha subunit	FdsA	Rmet_0555	R	YP_582710.1	-	47	8	2.0	7.7E-03
Other										
CHROM2	general stress response protein	CsbD	Rmet_5008	S	YP_587139.1	-	34	2	2.1	3.3E-02
CHROM1	putative type-4 fimbrial biogenesis protein*[2]	PilY1	Rmet_0192	NUW	YP_582347.1	x	241	5	3.1	8.1E-04
CHROM1	isocitrate lyase*[2]	AceA	Rmet_1385	C	YP_583537.1	x	113	5	4.6	8.5E-04
CHROM2	alkaline phosphatase*[2]	PhoA1	Rmet_4084	P	YP_586220.1	x	1062	19	3.1	3.1E-03
CHROM2	acid phosphatase*[2]	AcpA	Rmet_4809	M	YP_586940.1	x	69	3	9.1	1.8E-03
CHROM1	KAP P-loop containing ATPase protein	-	Rmet_2164	R	YP_584312.1	x	85	13	2.1	7.7E-03
CHROM1	ATPase-like protein	-	Rmet_3356	T	YP_585497.1	x	43	7	4.6	1.1E-02
CHROM1	P-loop-containing ATPase protein	-	Rmet_0297	R	YP_582452.1	-	79	3	10.1	3.9E-03
CHROM1	chromosome segregation ATPase	Smc	Rmet_1426	D	YP_583578.1	-	187	14	2.1	3.9E-02

CHROM1	D-tyrosyl-tRNA(Tyr) deacylase	Dtd	Rmet_0418	J	YP_582573.1	-	25	2	4.5	3.1E-02
CHROM2	putative metallo-dependent amidohydrolase*[2]	-	Rmet_5313	R	YP_587441.1	-	91	4	2.1	1.6E-03
CHROM2	4-hydroxybenzoate 3-monooxygenase	PobA	Rmet_4018	HC	YP_586155.1	-	18	3	2.7	4.5E-02
CHROM1	Acyl-CoA synthetase (AMP-dpendent)	-	Rmet_1061	I	YP_583216.1	-	56	6	2.4	4.9E-04
CHROM2	acyl carrier protein	AcpP	Rmet_4378	IQ	YP_586512.1	-	70	9	2.0	3.8E-02
CHROM1	fatty acid desaturase	-	Rmet_0888	I	YP_583043.1	-	53	6	9.7	2.2E-02
pMOL30	insertion element protein	TnpA	Rmet_5954	L	YP_145615.1	x	73	10	2.9	3.8E-03
CHROM1	carboxypeptidase G2 precursor	-	Rmet_0024	E	YP_582179.1	-	42	4	926.0	4.8E-03
CHROM2	conserved hypothetical protein	-	Rmet_5391	S	YP_587519.1	-	41	2	2.1	1.6E-02
CHROM1	conserved hypothetical protein	-	Rmet_0978	S	YP_583133.1	x	26	4	4.4	1.4E-02

Differentially expressed proteins were considered biologically significant with an average intensity ratio of at least two-fold and a *p*-value less than 0.05 if detected with two or more peptides per protein with a MASCOT identification score greater than 20. Bold locus tags highlight proteins in which more than one gene from the operon is differentially regulated. *[1] Fold changes >500 result from lack of detection in one condition. *[2] Also observed to be of lower abundance in MM284 at pH 8. Differentially expressed proteins were considered biologically significant with an average intensity ratio of at least two-fold and a *p*-value less than 0.05 if detected with two or more peptides per protein

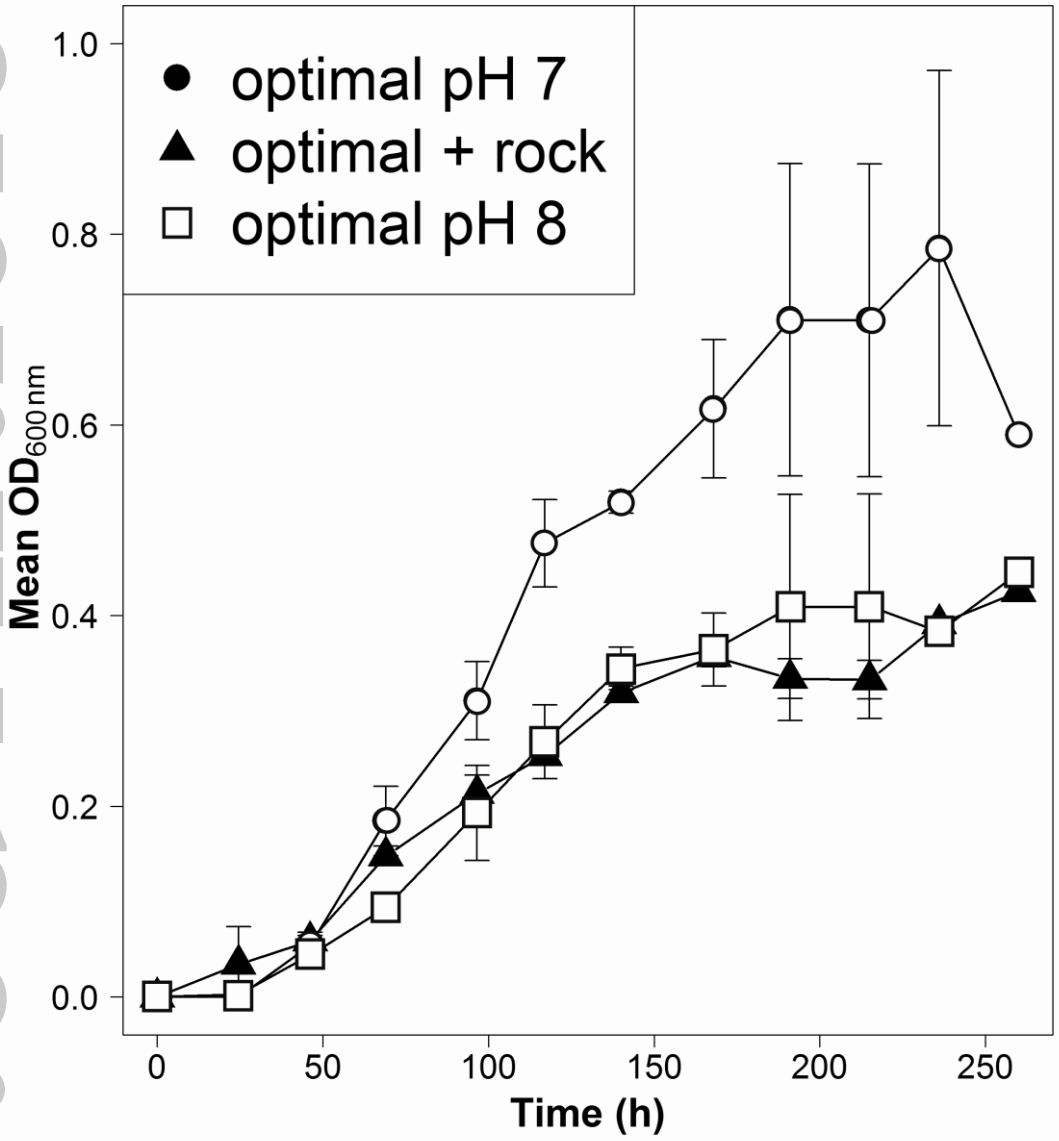
with a MASCOT identification score greater than 20. Column “X = common to all with rock” indicates whether the abundance of this protein was also lower in the other media types with rock added i.e. minus Fe + rock and minus Mg + rock.

621



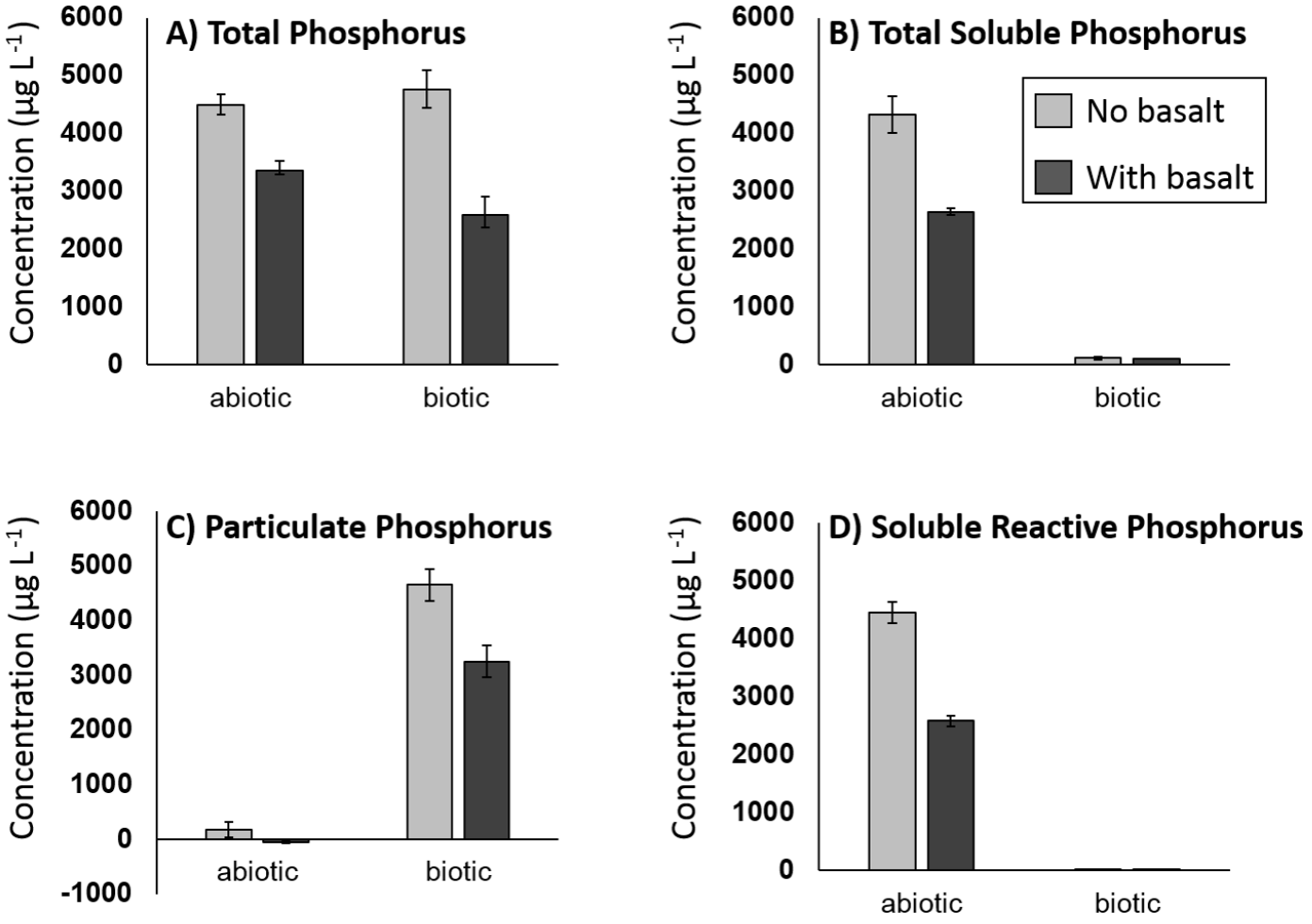
622

623 EMI_13093_F1



624

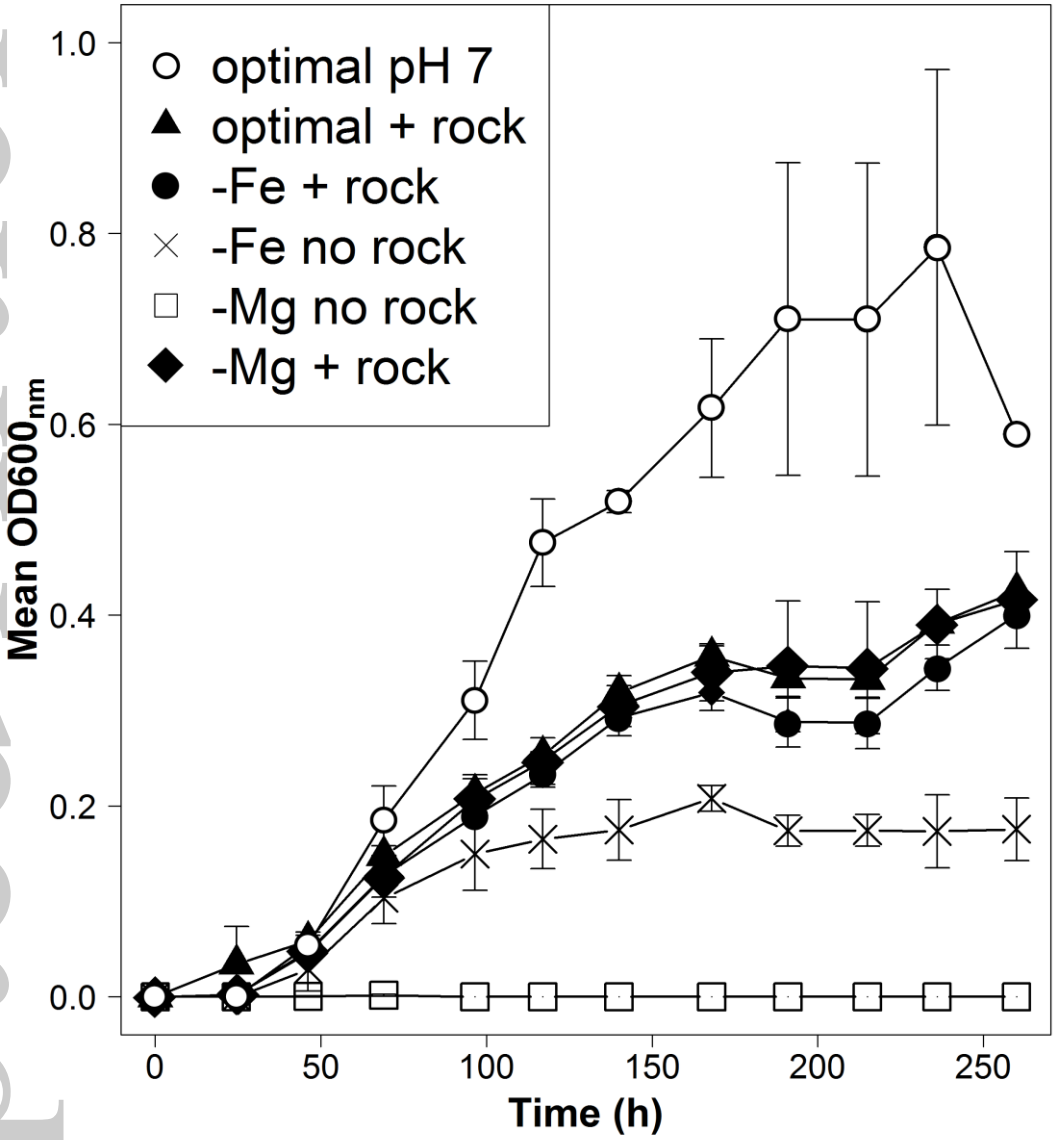
625 EMI_13093_F2n



626

627 EMI_13093_F3

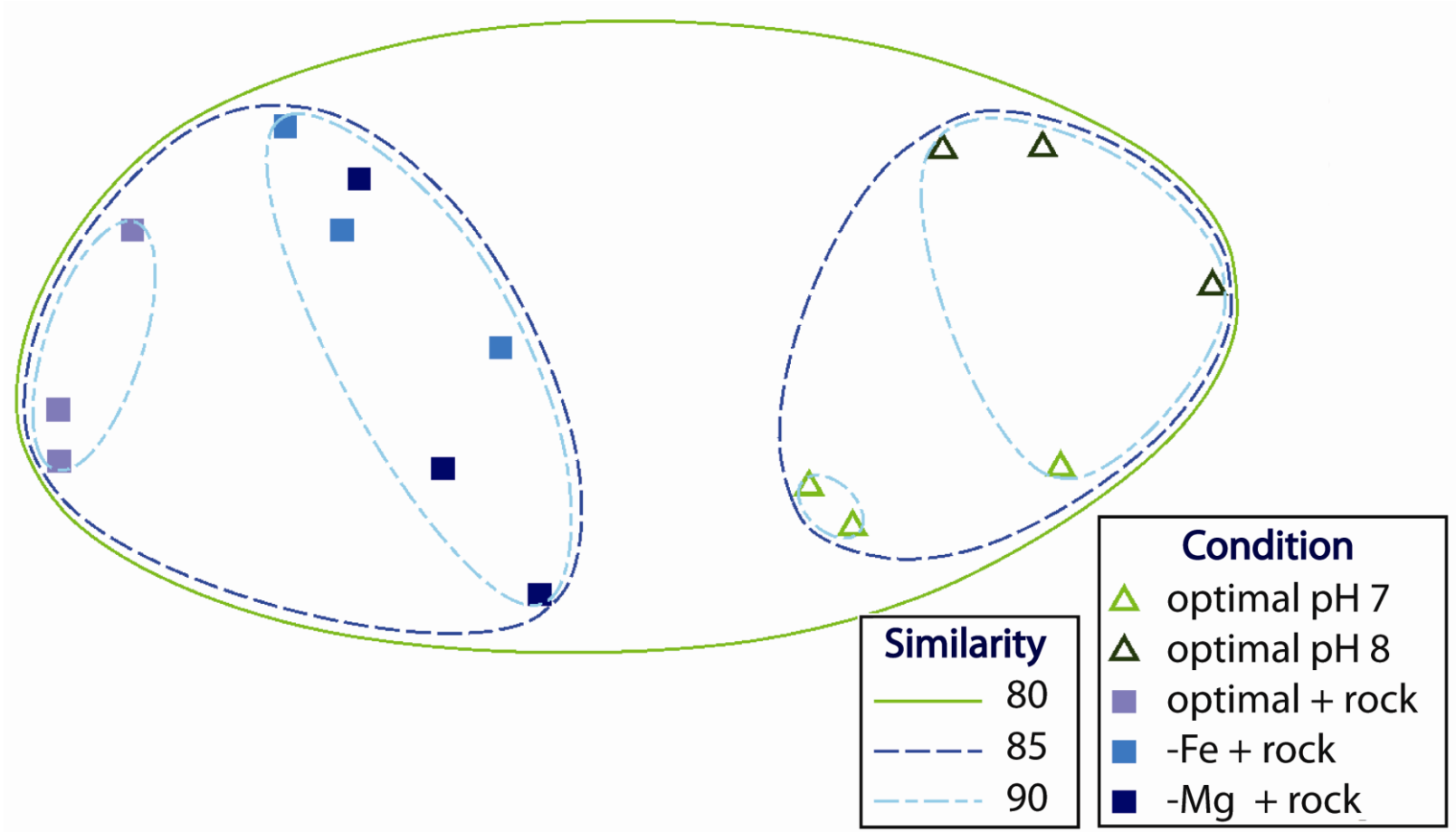
628



629

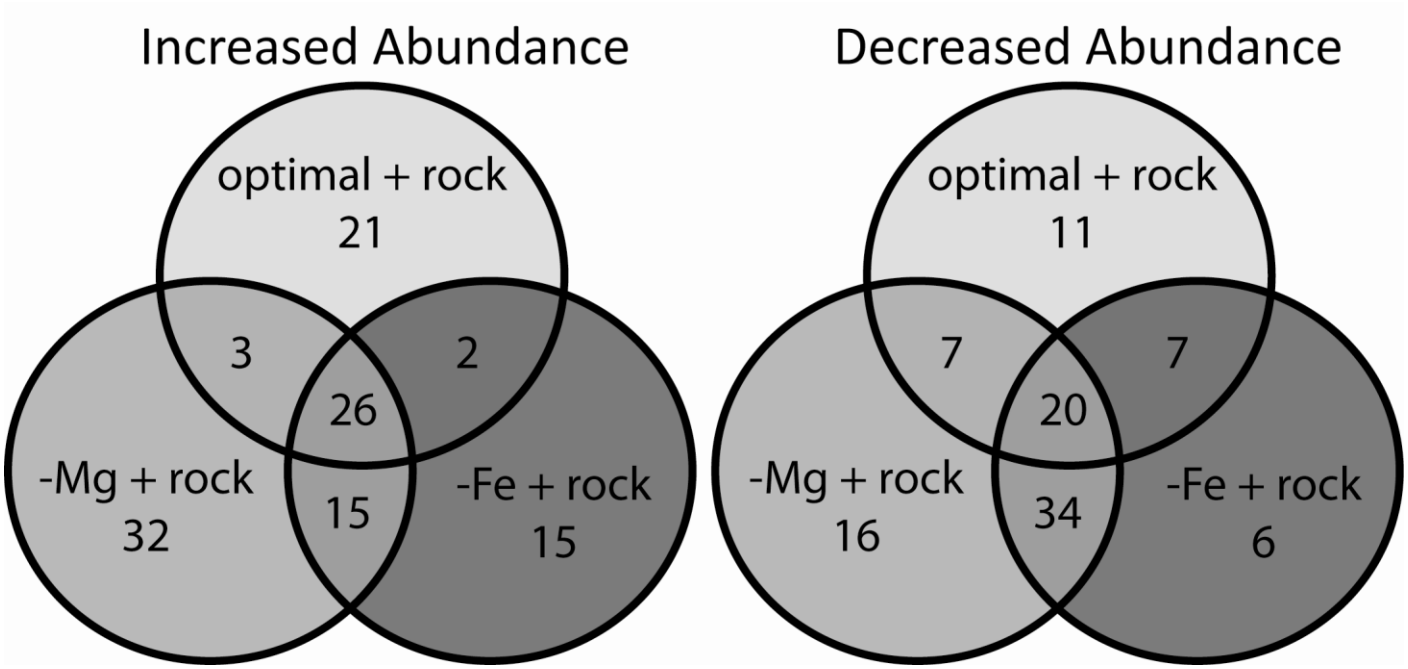
630 EMI_13093_F4new

631



632

633 EMI_13093_F5



634

635 EMI_13093_F6new