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Antagonistic co-limitation through ion promiscuity – On the metal sensitivity of *Thalassiosira* *oceanica* under phosphorus stress.

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Abstract

Nutrient limitation of primary producers is a fundamental principle in biogeochemical oceanography and has been used with great success in prescribing understanding to patterns of marine primary productivity. In recent years the paradigm of nutrient limitation has expanded from single nutrient limitation towards concepts of co-limitation by multiple resources. Interactive effects between multiple limiting resources are now thought commonplace in marine microbial communities. Here we investigate the response exhibited by phosphate-limited *Thalassiosira oceanica* to elevated concentrations of the phosphate analogs vanadate, arsenate and molybdate. Enrichments in external arsenate and vanadate to phosphate-limited cultures act to suppress growth rates entirely, an effect not seen in phosphate replete conditions. Retardation of growth rates is attributed to mistaken uptake through ion promiscuity as evidenced by observations of significant intracellular accumulation of both arsenic and vanadium under phosphate limited conditions. We describe this novel co-limitation scenario as dependent antagonistic co-limitation (DAC), and suggest that this phenomenon of non-deliberate intracellular accumulation could be used as both a proxy of phosphate stress in the modern ocean and a possible marker of phosphate depletion limiting the duration of oceanic anoxic events.

Abbreviations:

ICP-MS - inductively coupled plasma mass spectrometry

Pi - inorganic phosphate

ES - elemental stoichiometry

P_{IC} - intracellular phosphorus

As_{IC} - intracellular arsenic

V_{IC} - intracellular vanadium

Mo_{IC} - intracellular molybdenum

SOW - Synthetic ocean water

μMax - Maximal growth rate

FIAS - flow injection autosampler

DOP - dissolved organic phosphorus

GOE - great oxidation event

ERC - European Research Council

APPELS - A Periodic Probe of Elements in the Sea

1. Introduction

Biological growth is, in essence, a chemical process requiring an abundance of both chemical resource and a source of energy. A mismatch between the required and the available stoichiometry of chemical elements is the fundamental principle of nutrient limitation and is a cornerstone of ecological stoichiometry (Stern and Elser 2002). Nutrient limitation can be caused by a single nutrient where its deficiency causes suppression in growth rates (sensu Blackman limitation (Blackman 1905)) or can provide an upper limit on the amount of new biomass that can be formed (sensu Liebig limitation (de Baar 1994)). It can also take the form of co-limitation whereby two or more nutrients can simultaneously affect the growth rate or yield of a population. Here we investigate the potential for antagonistic effects experienced by the diatom *Thalassiosira oceanica* grown under sustained phosphate limitation in the presence of the oxyanions of similar geometry to phosphate - vanadate, arsenate and molybdate.

Phosphorus (P) is a component of both nucleic acids and lipids and therefore is a fundamental requirement for life. In oceanic surface waters, P is present in many forms including inorganic phosphate (PO_4^{3-} , henceforth referred to as *Pi*), which is readily bioavailable to most microbial species. P is also organically bound in forms such as phospho-esters and phosphonates, and reduced forms such as phosphite or hypophosphite (McGrath et al., 2013; Pasek et al., 2014; Figueroa and Coates 2017); all of

which are known to be bioavailable to at least some marine microbes (Dyhrman et al., 2006; Martinez et al., 2012; Polyviou et al., 2015). Despite this myriad of different P pools and the significant arsenal of P uptake strategies, there are vast regions of the surface ocean where P_i concentrations are in the low nanomolar concentration; here phytoplankton growth is believed to be constrained by P as a limiting resource (Karl 1999; Mather et al., 2008; Krom et al., 2010).

The speciation of P_i present in seawater is predominantly that of the P(V) oxyacids HPO_4^{2-} and $H_2PO_4^-$. Comparably in dilute solutions, at seawater pH, the dominant species of vanadium (V) and arsenic (As) are that of the +V oxidation state, phosphate-like, oxyacids mononuclear vanadate and arsenate, of the form $-H_nXO_4^{n-3}$ (Figure 1) (Cutter et al., 2001; Wang et al., 2009). Based on thermodynamics the dominant molybdenum (Mo) species in seawater is the +VI species MoO_4^{2-} (Baes and Mesmer 1976) with a small amount of the +V species reported (Bertine 1972; Brookins 1988). The anions vanadate, molybdate and arsenate, are often seen as analogous to phosphate (Crans et al., 2004). They are isoelectronic and isostructural with that of the phosphate ion. Oxygen bonds to the P/V/As/Mo in a tetrahedral sp^3 arrangement with bond lengths of 1.65 Å (V=O), 1.75 Å (Mo=O) and 1.73 Å (As=O) that are similar to the 1.55 Å of P=O in phosphate (Lee 1982; Wolfe-Simon et al., 2011). The ionic species of these three trace metals closely resembles the structure of the phosphate anion and hold the potential to interfere with phosphate biogeochemistry.

Mo, V and As are some of the most abundant trace metals in surface ocean waters with respective modern-day concentrations of Mo ~100 nM (Morris 1975; Sohrin et al., 1998; Ho et al., 2018), V ~35-45 nM (Morris 1975; Emerson and Huested 1991; Ho et al., 2018) and As ~20 nM (Cutter et al., 2001). These concentrations are high enough to regularly exceed those of P_i in surface waters in regions such as the Mediterranean Sea and the sub-tropical Atlantic (Karl 1999; Wu et al., 2001; Mather et al., 2008; Krom et al., 2010, Snow et al., 2015). Molybdenum displays a mostly conservative depth profile while vanadium and arsenic appear nutrient like with surface water depletion and relative enrichment at depth (Cutter et al., 2001; Ho et al., 2018).

V, Mo and As have been historically understudied in the marine realm, due in part to their mostly conservative distribution and assumed lack of influence in marine primary productivity; but recent studies have brought some interesting observations to the fore. Numerous instances of intracellular As, V and Mo accumulation have been observed. *Trichodesmium* sp., a diazotrophic cyanobacterium has been shown to have elevated concentrations of cellular V (Tovar-Sanchez and Sanudo-Wilhelmy 2011; Nuester et al., 2011; Snow 2014), As (Snow 2014) and Mo (Nuester et al., 2011; Snow 2014) - all observations from conditions where P_i stress is a potential factor. Additionally, Nalewajko et al., (1995) observed a 7.5-fold increase in intracellular V concentrations of *Scenedesmus* cultures grown under elevated V conditions. A pair of studies have recently demonstrated

an environmental correlation between dissolved V concentrations and phytoplankton primary productivity in the Atlantic Ocean. Klein et al., (2013), used linear regression modelling of a suite of environmental parameters to demonstrate a strong correlation between chlorophyll a, biogenic Si and dissolved V concentration in the North Atlantic spring bloom. They suggested diatom uptake may be responsible for the correlation. Pinedo-Gonzalez et al., (2015), meanwhile found V and Mo, alongside NO_3^- , to be variables that strongly associated with primary production in the Atlantic and found a similar relationship in the Pacific between V, PO_4 and Fe. Further evidence of a possible link between V uptake and diatom abundance was presented by Osterholtz et al., (2014). In this study, a diverse cohort of diatom strains all drew-down concentrations of dissolved vanadium across the organism's growth cycle with particular depletion occurring in the late exponential and stationary growth phases (Osterholtz et al., 2014).

Molybdenum is an essential micronutrient thought to be required by a majority of marine microbes and is present in upwards of 60 different enzyme classes (Wang 2012 and references therein). It is an important metal for the nitrogen cycle due to being present in nitrogenase, nitrate reductase and nitrite oxidoreductase and therefore is involved in the processes of nitrogen fixation and assimilatory nitrate reduction (Stiefel 1996; Moreno-Vivian et al., 1999; Maia et al., 2017). The essentiality of molybdenum in a wide range of enzymes may explain some of the intracellular accumulation

observed in the literature. However, vanadium's biological utility is less widespread amongst marine microbes - two V containing enzymes of note are those of the alternative vanadium nitrogenase and vanadium haloperoxidases (Crans et al., 2004). V-nitrogenase is thought unlikely to be widespread in the contemporary oceans due to Mo deficiency appearing to be a prerequisite to its invocation (Rehder 2000), therefore V-nitrogenase seems an unlikely cause of the observed V accumulation (Tovar-Sanchez and Sanudo-Wilhelmy 2011; Nuester et al., 2011; Snow 2014). V-haloperoxidases are commonly present in the genomes of marine microalgae (Crans et al., 2004) including *Thalassiosira* species (Hughes and Sun 2016). Arsenic meanwhile has an actively debated biological importance with many suggesting that it has potential as a functional replacement for cellular P (reviewed in Tawfik et al., 2011). However, little proof exists of a deliberate biological function for As in marine phytoplankton and so explanations of observed cellular accumulation are lacking.

The chemical similarity of these oxyacids has been demonstrated to pose a challenge for phosphate-limited microbial communities; the non-specific uptake of arsenate and vanadate via phosphate uptake channels has been reported in the past (Hellweger et al., 2003; Nalewajko et al., 1995b). Uptake, reduction and methylation before subsequent excretion of arsenate to dimethylarsinic acid has been shown to occur as both a function of P_i availability but also presumed P_i uptake during early-bloom conditions

(Hellweger et al., 2003). Similarly, the degree of photosynthetic inhibition caused by V enrichment was observed to be affected by P_i availability of freshwater phytoplankton with P-deficient cells being more acutely affected by vanadium inhibition (Nalewakjo et al., 1995b). A commonality between these studies is the speculation that the inhibitory effect of either As or V is at least partially attributable to non-specific uptake of AsO_4^{3-} or VO_4^{3-} via PO_4^{3-} uptake pathways.

There has been as yet, no comprehensive assessment of the above-described ion promiscuity, seeking to compare the potential additive or antagonistic effect that these three abundant oxyacids might play in the nutrient uptake of P-deficient marine phytoplankton. To this end, we present an element enrichment data set demonstrating the effect of vanadate, arsenate and molybdate on the growth of *Thalassiosira oceanica* CCMP1003, an open ocean diatom isolated from the P deplete Sargasso Sea. We assess the potential nutritional role of these elements and reflect on the modern and paleo-oceanographic implications of our findings; to advance understanding of antagonistic co-limitation, cellular elemental homeostasis and the role they play in the marine microbiome.

2. Methods

2.1 Culturing and Media amendments:

Thalassiosira oceanica strain CCMP1003 was grown in chelexed Aquil* synthetic ocean water (SOW) (Price et al., 2005) in a 20 °C incubator with 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light operating on a 12:12 hr light:dark cycle. Two different phosphate regimes were implemented, phosphate replete where 10 μM PO_4^{3-} was added and phosphate deplete where 100 nM PO_4^{3-} was added. Triplicate cultures of *T. oceanica* were established in acid-cleaned (5% HNO_3^{2-} for 24 hours), and UV sterilised 24-well plates (Corning Costar). Each culture comprised 2 ml of fresh media and 50 μL inoculum. Vanadium, Arsenic and Molybdenum were added in the form of sodium orthovanadate (Na_3VO_4), sodium arsenate (Na_3AsO_4) and sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) respectively. Metal additions were made as listed in Table 1.

Chemical modelling of media amendments using VisualMINTEQ showed the additions of arsenate, vanadate and molybdate resulted in the intended concentrations of the desired chemical species (Figure 1). Arsenate additions resulted in the formation of HAsO_4^{2-} , H_2AsO_4^- and AsO_4^{3-} which together represented approx. 99% of the Arsenic added (Figure 1). Vanadate additions resulted in the formation of H_2VO_4^- and HVO_4^{2-} which together accounted for approx. 99% of the Vanadium added (Figure 1). Molybdate

additions resulted in the formation of MgMoO_4^{2-} and MoO_4^{2-} which accounted for approx. 95% of the molybdate added. P_i in the media was approximately equally distributed amongst NaHPO_4^- , MgHPO_4 and HPO_4^{2-} which together accounted for 85% of the P addition (Figure 1). P_i was present in excess of arsenate, vanadate or molybdate in each of the P_i -replete culture treatments except for the 10,000 nM treatment. Under P_i -deplete conditions, the concentration of P_i in the media was always less than the arsenate, vanadate or molybdate additions except for the no addition control.

2.2 Culture Growth Assessment:

A TECAN Spark® Multi-mode plate reader (TECAN Trading AG, Switzerland) was used to assess relative chlorophyll fluorescence at the same time every day of the experimental period. In-vivo chlorophyll fluorescence was measured by inserting the entire 24-well culture plate into the plate reader, allowing for a non-destructive, non-invasive measurement that limits the risk of trace metal contamination. The 24-well plate was shaken linearly for 2 minutes at an amplitude of 2 mm and frequency of 810 rpm. In-vivo chlorophyll fluorescence was measured with an excitation wavelength of 485 nm and emission wavelength of 680nm, the fluorescent gain was kept consistent across each experimental run and was either set to 60 or 80 depending on the experiment.

Maximum specific growth rate (μ_{Max}) was calculated using the slope of the linear phase of semi-log plots of relative chlorophyll fluorescence over time. The linear fit utilised at minimum three consecutive days of fluorescence data. Statistical significance for differing specific growth rate between culture conditions was assessed by one-way analysis of variance (ANOVA, $p < 0.05$) using GraphPad Prism 7. Relative growth rate within a range of metal concentrations was calculated as the percentage decrease relative to highest μ_{Max} observed for that experiment.

2.3 Measuring Cell Quotas by ICP-MS

A series of cultures with differing P and V/As/Mo treatments were established as described above, and analysis was conducted for the determination of intracellular P, V, As and Mo concentration.

At mid- to late exponential phase of growth, cell abundance and size was determined using a Coulter Counter Z2 cell counter. Cultures were then transferred to trace-metal clean centrifuge tubes under laminar flow before centrifugation at $2000 \times g$, 15°C for 20 minutes. Two wash procedures were used to remove residual media and loosely bound surface metals. Each wash consisted of the addition of 15 ml chelex cleaned synthetic ocean water (to which no macronutrients or trace metals were added), vortexing to resuspend cells then centrifuged at $2000 \times g$ 20°C for 5 minutes. The washed cell pellet was kept frozen at -80°C until digestion.

Due to the presence of viable cells, detrital material and authigenic oxyhydroxides, the biogenic fraction of harvested material is typically operationally defined (Ruaschenberg and Twining 2015). Cell washing or leaching techniques such as by weak acids (Berger et al., 2008) or ligand-promoted dissolution (Tovar-Sanchez et al., 2003; Tang and Morel 2006) have been employed by others to remove lithogenic and authigenic contributors to the overall metal concentration in this type of harvested material. In this study, we have opted to forego these steps because the hypothesised non-deliberate uptake of P analogues brings with it significant uncertainty regarding in which operationally defined pool the V, Mo or As may exist. Therefore, we define our cellular metal concentrations as that which reflects truly intracellular biogenic material but also must include potential surface-adsorbed material of authigenic origin (due to all work having been carried out in rigorously cleaned and air filtered laboratories the lithogenic fraction is thought to be very small and therefore is not considered during interpretation).

Acid digestion consisted of overnight reflux in a 3:2 ratio solution of quartz distilled HNO_3^- (in-house production) and H_2O_2 (ROMIL UpATM) at 150 °C. Following complete digestion, the samples were evaporated to dryness before being resuspended in 5 ml 2% HNO_3^- ready for ICP-MS analysis. Triplicate sample, blank and certified reference material BCR-414 were digested, and metal recoveries were similar to those presented in Zhang et al., 2018.

Cellular metal quotas were measured by inductively coupled mass spectrometry (ICP-MS) using a Perkin Elmer Nexion 350D (Perkin Elmer, Warrington, UK) interfaced with an Elemental Scientific Flow Injection Automation System (FIAS) using the method detailed in Zhang et al., 2018; modified such that only P, V, Mo and As were measured.

3. Results

3.1 Phosphate, vanadate, arsenate and molybdate inhibition of growth

Control cultures, where no V or As were added and Mo was maintained at the standard 100 nM for Aquil* media, showed a significant depression in growth rate (t-test, $p < 0.05$) between the 'Pi-deplete' concentration of 100 nM to the 'Pi-replete' concentration of 10 μ M. Here average μ_{Max} of 0.39 ± 0.18 and $0.55 \pm 0.11 \text{ day}^{-1}$ were observed for the respective treatments. Such consistently lower growth rates between replete and deplete conditions confirms the intended P limited conditions for the Pi-deplete cultures (Figure 2).

When Pi was replete, *T. oceanica* had a growth rate that ranged from $0.48 - 0.78 \text{ day}^{-1}$. Across the assessed concentration ranges of As, V and Mo (0 - $10 \mu\text{M}$ [Me]) there was no significant change in maximal growth rate when Pi was abundant, suggesting either a lack of biological requirement for V, As or Mo or more likely that trace concentrations in the basal media were sufficient to meet the cells demand in these 'no addition' experiments (Figure 2). Equally, there was no observed inhibition of growth rate at elevated As, Mo or V concentrations suggesting toxicity from these metal additions has no significant effect on growth rate when phosphate is replete.

Conversely, when Pi was limiting, all maximum growth rates were

observed to be suppressed relative to their *Pi*-replete counterparts. Under these culture conditions, μ_{Max} never exceeded $0.53 \pm 0.05 \text{ day}^{-1}$ for any of the *Pi*-deplete cultures (Figure 3). No statistically significant growth rate variation was observed across the range of Mo additions under *Pi*-deplete conditions (Figure 2). Mo was not detrimental to cells even when present at two orders of magnitude higher than present-day environmental concentrations. No increase in growth rate was observed for any culture where Vanadium was added, either under *Pi*-replete or deplete conditions, suggesting the addition of V to these cultures provided no nutritional benefit to *T. oceanica*. Vanadium concentrations above $1 \mu\text{M}$, however, caused total retardation of growth in all *Pi*-deplete cultures assessed (Figure 2). Addition of As did not stimulate growth in either *Pi*-replete or deplete conditions and As did not retard growth under *Pi*-replete conditions (Figure 2). However, addition of As beyond environmental concentrations of 20 nM to *Pi*-deplete cultures invoked significant retardation in growth rates. At 100 nM [As] growth rates dropped to just 20% of μ_{Max} any conditions with $[\text{AsO}_4^{3-}] > 100 \text{ nM}$ did not support *T. oceanica* growth (Figure 2). Overall, As, Mo and V have no impact on *Pi*-replete cell growth, but As and V are detrimental under *Pi*-deplete conditions.

3.2 Cellular Metal Quotas

3.2.1 Phosphate Deplete Cultures

Given the observations of antagonism of growth of *Pi*-deplete cultures in the presence of As and V, we might expect a disproportionate cell accumulation of V and As in those cells compared to *Pi*-replete cells. To this end, larger volume cultures of elevated, but not growth rate inhibiting, V, As and Mo concentrations (500nM, 20nM and 500nM respectively) were grown and analysed for intracellular accumulation. Mean μ_{Max} varied between 0.95 - 1.03 day⁻¹ but showed no statistically significant differences between control and treatment cultures (Student's t-test, n=3, P<0.05) (Figure 3). We note that these large volume cultures presented elevated maximal growth rates compared to small volume, microplate-based cultures and suggest that this difference may arise from to the different experimental setups. We only compare growth rates between conditions within a similar experimental setup i.e. from internally consistent setups, so this difference should not affect our results. Intracellular phosphorus (P_{IC}) concentrations were invariant between metal treatments for *Pi*-deplete cultures, ranging from 0.54 - 2.33 fmol cell⁻¹ (Student's t-test, n=3, P<0.05) (Figure 4). Cell Mo concentration were the same for cells grown with no addition of Mo and 500 nM Mo. Relative to *Pi*-deplete cultures with no metal addition, a statistically significant enrichment of As_{IC} and V_{IC} was observed when grown under elevated concentrations of the respective

metals (Figure 4). We observed V_{IC} concentrations reaching 406 ± 238 amol cell⁻¹ and As_{IC} concentrations reaching 8.81 ± 2.22 amol cell⁻¹ when exposed to elevated external concentrations of Vanadate and Arsenate. Such cellular enrichments without significant P_{IC} enrichment resulted in the cellular V:P and As:P increasing from 0.67 to 173 (V) and 0.04 to 16.1 (As) mmol mol⁻¹. Here both enriched cultures had intracellular concentrations far exceeding that of the external environment M:P ratios of 0.2 As:P and 5 V:P.

3.2.2 Phosphate Replete Cultures

Pi sufficient cells were observed to accumulate significant amounts of V_{IC} when $[VO_4^{3-}]$ was very high (100 μ M). Such accumulation is comparable to that observed for *Pi*-deplete cells grown in 500 nM $[VO_4^{3-}]$ (Figure 4). *Pi*-replete *T. oceanica* cultures grown under 0, 10 and 100 μ M $[VO_4^{3-}]$ displayed no significant change in growth rate and no change in per cell P_{IC} concentration with values ranging from 12.42 - 13.78 fmol cell⁻¹. However, under 0 and 10 μ M [V] growth conditions V_{IC} was observed to be <1 amol cell⁻¹ compared with 2.58 fmol cell⁻¹ when grown under 100 μ M $[VO_4^{3-}]$ (Figure 4C). Such an enrichment represents a 2500-fold increase in V_{IC} compared to a 10-fold increase in external concentration and therefore is suggestive of an active process. Interestingly the concentration of Mo_{IC} observed under 100 μ M $[VO_4^{3-}]$ growth was lower than that observed in either 0 or 10 μ M culture; however, this was not a statistically significant observation.

3.2.3 Cell Size normalised Vanadium enrichment.

The average diameter of *Pi*-replete cells was $6.77 \pm 0.27 \mu\text{m}$, while *Pi*-deplete cells were approximately 19% smaller at $5.46 \pm 0.30 \mu\text{m}$. Assuming a spherical cell, this results in a 48% drop in cell volume. Normalising P_{IC} and V_{IC} quotas to these cell volume estimates show a substantial drop in P_{IC} concentration from 81 mM to 22.85 mM upon *Pi* stress (Figure 5A). Under elevated external $[\text{VO}_4^{3-}]$ a substantial elevation is observed for V_{IC} concentration: *Pi*-deplete cultures grown in the presence of 500 nM $[\text{VO}_4^{3-}]$ showed an increased V_{IC} from $<0.01 \text{ mM}$ to 5.36 mM, while under *Pi*-replete cultures an increased V_{IC} concentration is not seen until $[\text{VO}_4^{3-}]$ external is $>10 \mu\text{M}$. At 100 μM external $[\text{VO}_4^{3-}]$, V_{IC} concentrations of 18.34 mM were observed. Such V_{IC} enrichments represent 23-24% of P_{IC} , albeit at vastly different external $[\text{VO}_4^{3-}]$. Although not statistically significant, it is interesting to note the modest elevation in intracellular P_{IC} concentration when V_{IC} is elevated under both *P* replete and deplete conditions (Figure 4C).

4. Discussion

4.1 Co-limitation through ion promiscuity

Pi is often found at very low concentrations in the environment and is cited as a growth-limiting factor for phytoplankton in significant regions of the world's oceans (Karl 1999; Mather et al., 2008; Krom et al., 2010). Eukaryotic and prokaryotic microbes have evolved access to numerous alternative P pools to alleviate such resource scarcity (Dyhrman et al., 2006; Martinez et al., 2012, Polyviou et al., 2015). Despite this enzymatic arsenal of P acquisition strategies, *Pi* remains the most easily accessible form of P to many marine microbes. Therefore, cellular attempts at acquiring *Pi* persist even when extracellular conditions are severely *Pi* limited and alternative sources of P are called upon (Jansson et al., 1988). Moreover, the utilisation of complex organic forms of phosphate must first be preceded by external liberation of *Pi* so any processes that interfere with the uptake of orthophosphate would likely also affect DOP acquisition strategies (Cembella et al., 1984; Jansson et al., 1988).

Our observations of acute vanadate and arsenate toxicity, only under *Pi* stress conditions, presents a novel condition where the elevated concentration of a secondary substance actively exacerbates *T. oceanica's* pre-existing *Pi* stress; resulting in complete retardation of growth (Figure 2). A relatively modest 4-fold increase in $[\text{AsO}_4^{3-}]$ from 25 nM to 100 nM

concentration was sufficient to reduce *T. oceanica*'s growth rate by 80% when *Pi* was limiting but had no effect under *Pi*-replete conditions. A more substantial increase in $[\text{VO}_4^{3-}]$ concentration from the approximate environmental concentration of 40 nM to >1 μM inhibited *Pi* stressed *T. oceanica* growth entirely. Neither growth rate suppressing effect was observed under *Pi*-replete conditions even when $[\text{VO}_4^{3-}]$ and $[\text{AsO}_4^{3-}]$ were further increased. We, therefore, suggest the growth-limiting effects of arsenate or vanadate relates to the *Pi* nutritional status of *T. oceanica* through a process of ion promiscuity: vanadate and arsenate are inadvertently picked up and internalised by *T. oceanica* in its efforts to acquire phosphorus. Such mistaken uptake maybe present as direct V toxicity but also could be exhausting the cells capacity to acquire *Pi*, thus, presenting as a severe *Pi* stress response.

A typical planktonic response to *Pi* stress is to increase *Pi* uptake, either by invoking a high-affinity *Pi* transport system or by increasing expression of low-affinity transport proteins. The specific *Pi* uptake pathway induced by *T. oceanica* is not currently known. However, Perry 1976 demonstrated that *Pi* stress in the closely related diatom *Thalassiosira pseudonana* triggered an increase in maximal uptake rate rather than an increase in affinity - suggesting up-regulation of a low-affinity *Pi* uptake pathway. Transcriptomic and proteomic analysis of *Pi* stressed *T. pseudonana* revealed this low-affinity transporter as being THAPS_24435 (Dyhrman et al., 2012)

of which *T. oceanica* CCMP1005 has a homologous protein in THAOC_09588. We, therefore, speculate that if *T. oceanica* lacks a high-affinity *Pi* transporter, then it has no apparent means of increasing *Pi* selectivity under *Pi* stress and instead relies on the increased usage of the low-affinity pathway and other non-*Pi* uptake strategies (i.e. DOP, reduced P). The low-affinity *Pi* uptake pathway can then effectively deplete the near-cell extracellular environment of *Pi* to a far greater extent than under *Pi*-replete conditions. This results in increased ion misidentification or promiscuity, which may inadvertently introduce VO_4^{3-} and AsO_4^{3-} into the intracellular space.

Nutrient co-limitation can take many forms, and there are many definitions in the literature (Arrigo et al., 2005; Saito et al., 2008; Allgeier et al., 2011; Moore et al., 2013). The majority of co-limitation studies focus on additive or super-additive scenarios where addition of two (or more) nutrients is able to stimulate growth and produce an effect equal to (additive, Figure 6A) or greater than (super-additive, Figure 6B) the sum of the individual stimuli if added in isolation (Allgeier et al., 2011). Sub-additive or antagonistic co-limitation describes scenarios in which addition of multiple nutrients produces an effect less than the sum of the nutrients if added independently (sub-additive, Figure 6C) or addition of a secondary nutrient acts to suppress the stimulatory effect that one or both nutrients have if added individually (antagonistic, Figure 6D). This framework of

(co)-limitation results from describing the action produced by the addition of a stimulating resource. If the addition of resource R_1 is capable of stimulating a population, then the initial, unperturbed, population must have been deficient in resource R_1 . A population exists in a state of deficiency of resource R_1 and so can be described as being ' R_1 limited'. A state of limitation is, therefore, dependent on two factors - the absolute abundance of a resource and the organism's ability to access that resource. We suggest that the antagonism suggested here, brought on by the presence of excess AsO_4^{3-} or VO_4^{3-} under Pi -deplete conditions, provides a mechanism by which *T. oceanica*'s ability to access Pi is reduced.

Consequently, a population of *T. oceanica* growing under Pi -deplete conditions will have the severity of P limitation modulated by the presence or absence of other anions - this is a response which is distinct from merely being toxicity. We term this 'dependent antagonistic co-limitation' (DAC) (Figure 6E). DAC can be considered distinct to Saito et al., 2003's 'biochemically dependent co-limitation'. Where uptake of one resource is dependent on the sufficient nutrition of a second. DAC, contrasts in that it requires an absence of the antagonist (Vanadate or Arsenate) to alleviate the stress. Co-limitation scenarios involving a process of dependent antagonism are poorly studied and, like sub-additive and antagonistic co-limitation (Alleger et al., 2011), may be more widespread in the surface oceans than currently thought.

4.2 Ecological stoichiometry and cellular homeostasis:

Ecological stoichiometry (ES) theory (Sternner and Elser 2002) is a well-established and often used framework for the interpretation of marine phytoplankton productivity and distribution (Redfield 1958; Martiny et al., 2013). Studying the energetic and chemical composition of an organism as it relates to ecological interactions - ES considers both the demand of the organism for chemical elements and its degree of elemental homeostasis. The majority of research into ecological stoichiometry as it relates to marine primary producers has been to focus, with great success, on macronutrients C, N and P (Redfield 1958; Geider and LaRoche 2002; Klausmeier 2004).

It is a well-documented and advantageous strategy for microbial organisms to hoard a potentially limiting resource when supplies are readily available. Phosphorus is known to be stored in polyphosphates (Martin et al., 2014), nitrogen in cyanophycin granules (Obst and Steinbüchel 2006) and iron in ferritin complexes (Marchetti et al., 2009). So-called luxury uptake strategies act as a safeguard against potential resource scarcity in the future and make the biological interpretation of a cell's elemental composition difficult. Indeed, the observations show a substantial increase in P_{IC} concentration under differing levels of P_i repletion, indicative of P_i luxury uptake. P_i -replete cells were observed to have P_{IC} concentrations ranging from 72-98 mM while P_i -deplete cells had concentrations of 15 - 31 mM - a decrease of 2.3 - 6.5 fold while maintaining cellular viability albeit at a lesser growth rate (Figures {growth_max} and

{enrichment}). It is likely that a portion of this P_{IC} in replete cells is as internal P stores acquired during luxury uptake. A further complication to interpreting cellular elemental composition is the notion that the composition of a cell is not as well controlled as previously thought, and that homeostatic capacity or strength is both element and organism dependent (Sternier and Elser 2002; Meunier et al., 2014; Jeyasingh et al., 2017). In this study we demonstrate substantial enrichment in cellular vanadium - with no observable effect on growth rate and no readily ascribed biological function; It seems unlikely that such significant enrichments in V_{IC} are due to the V-enzymes detailed previously. Under both *Pi*-replete and *Pi*-deplete conditions, albeit, at different $[VO_4^{3-}]$ concentration, we observed an accumulation of V_{IC} equal to approx. 17% of intracellular P concentrations. From these observations, we can interpret this enrichment as unintentional and a failure of cellular homeostasis efforts. Thus, the observed enrichment of V_{IC} can be interpreted under an ES framework, not as a chemical requirement but as a homeostatic trait; Where *T. oceanica* seemingly do not have adequate homeostatic control under conditions of excessive external $[VO_4^{3-}]$ relative to *Pi*. It may be that with no observed detrimental effect on growth rate, there is perhaps no compelling reason to select against $[VO_4^{3-}]$ uptake. Critical to this is the differential homeostatic capacity at varying degrees of *Pi* availability. *Pi*-deplete cultures show a broadly similar lack of V homeostasis at 500nM external $[VO_4^{3-}]$ as do *Pi*-replete cells at 100 μ M $[VO_4^{3-}]$ suggesting a 200-fold

increase in V-related homeostatic capacity under nutrient-replete conditions.

Under *Pi*-replete conditions *T. oceanica* is observed to enrich intracellular *Pi* $\sim 8.1 \times 10^3$ fold relative to external *Pi* availability (i.e. $[P]_{(IC)} / [P]_{(Media)}$). Conversely, under *Pi*-deplete conditions, this accumulation is $1.5 - 3.1 \times 10^5$. Such an enrichment indicates an 18-38-fold increase in P uptake under *Pi* stress conditions. An increase that agrees with previous reports of a 10-100-fold increase for *Pi* starved algae (Jansson 1988 and references therein). Meanwhile, V_{IC} enrichment showed a distinct *Pi* mediated response, under *Pi*-replete conditions V_{IC} enrichment was 0.5 for an external $[VO_4^{3-}]$ of 10 μM , suggesting strong cellular homeostasis under these conditions. Increasing $[VO_4^{3-}]$ to 100 μM for *Pi*-replete cells saw a moderate breakdown of V homeostasis, where intracellular enrichment was 183-fold higher than the external environment. Conversely, under *Pi*-deplete conditions external $[VO_4^{3-}]$ concentrations of 500 nM lead to a severe decrease in V cellular homeostatic capacity where intracellular concentrations were observed to be 1073 times higher than the external environment. These results suggest that homeostasis is maintained under *Pi*-replete conditions up to 10 μM $[VO_4^{3-}]$, but at 100 μM $[VO_4^{3-}]$ we see evidence of active, although not necessarily intentional, V transport into or onto the cell. This active transport is significantly greater under *Pi*-deplete conditions where a lower external $[VO_4^{3-}]$ (500 nM) triggers a far more

severe breakdown of cellular homeostasis. Such a significant increase in enrichment suggests these observations are not simply an accumulation of authigenic V containing particles; the modulating role of P_i concentration makes it clear that this is not a passive, abiotic process. Autotrophs are significantly more variable in their stoichiometry and have decreased homeostatic control relative to heterotrophs (Sterner and Elser 2002).

Further to this are observations that have established homeostatic capacity is inversely correlated to growth rate, where homeostatic control is diminished under sub-optimal growth rates (such as under P_i limitation) (Sterner and Elser 2002). We surmise that the P_i stress conditions present in the environment could result in the uptake and accumulation of both As and V without a deliberate biological function. This mistaken uptake, borne of a lack of uptake selectivity, could be the origin of the observations made by Pinedo-Gonzalez et al., 2015 - where surface water V concentrations correlated with PO_4^{3-} concentrations. Or the observations of Klein et al., 2013 where V correlated with biogenic Si and chlorophyll-a concentrations. Vanadate drawdown may, therefore, act as a proxy of surface water P stress amongst the primary producers, akin to that inferred for Cd uptake and the correlation between Cd and P in seawater (Horner et al., 2013).

5. Implications

5.1 Crude oil formation and usage:

Vanadium, alongside Nickel, is one of most abundant trace metals in crude oils and asphalts (Treibs, 1936; Erickson et al., 1954; Vine and Tourtelot 1970) with concentrations of ~1600 mg/kg (Barwise et al., 1990) and as high ~4800 mg/kg in bitumen-rich oils. The V (and Ni) are strongly bound in metal-organic complexes such as tetrapyrroles (Lewan and Maynard 1982) derived from transformations of organic porphyrin molecules such as hemes or chlorophylls (Lewan and Maynard 1982; Filby et al., 1987). Consequently, oils of high V concentrations are believed to originate from marine algal deposition (Lewan and Maynard 1982; Ocampo et al., 1986) with the highest V concentrations from algae that experience anaerobic conditions early in their depositional history (Lewan and Maynard 1982). The most likely sources of elevated V in algae originating deposits is from endemic V in the source material (cellular V), with the V content of interstitial waters (being the second most likely sources) believed to be too low to account for the observed enrichments (Lewan and Maynard 1982). We, therefore, propose a possible link between vanadium content of crude oils and the P sufficiency of the algal source material., Under conditions of P_i stress, we demonstrate significant enrichment in intracellular V_{IC} . Other studies have likewise observed environmental

enrichment of intracellular V (Tovar-Sanchez and Sanudo-Wilhelmy 2011; Nuester et al., 2011; Snow et al., 2014) under potentially P limiting conditions. Algal bloom conditions have been shown to modulate As uptake due to the presumed onset of *Pi* stress (Hellweger et al., 2003). It, therefore, seems likely that a similar modulation of inadvertent $[\text{VO}_4^{3-}]$ uptake might occur during diatom blooms - ultimately lead to a significant drawdown of V from the water column into the sediments and its incorporation into oil deposits. It is therefore tempting to speculate that such rich V accumulation is indicative of *Pi* limitation constraining the duration of the oceanic anoxic events (OAE). Adsorption and removal of P onto iron oxyhydroxides in a ferrous ocean (Handoh and Lenton 2003) could have provided a natural feedback to limit the OAE and with it bring about Vanadium drawdown. So Vanadium enrichment could act as a marker for such a mechanistic limit of OAE duration.

Due to increased use of these V rich petroleum products the human emissions of vanadium to the atmosphere have more than doubled in the space of two decades; these emissions presently exceed background emissions by a factor of 1.7 (Schlesinger et al., 2017). Further interest in petroleum derived from these unconventional deposits suggests this factor is expected to increase in the future (Schlesinger et al., 2017). Moreover, vanadium has the highest anthropogenic enrichment factor of all the trace elements in the atmosphere and changing societal demands has prompted calls for a reevaluation of V as a potential environmental hazard (Watt et al.,

2018). Increased anthropogenic mobilisation of V to the atmosphere is likely to increase the supply of V to the oceans and may act to further increase the V:P ratio of surface waters. Such a change in surface water V:P may serve to exacerbate *Pi* stress and limitation of the primary producers.

5.2 Palaeoceanographic implications

Cellular level protection against arsenic damage is positioned deeply in the tree of life, suggesting an evolutionarily early sensitisation to this metal (Duval et al., 2008). It is believed that the geological abundance of oxidised arsenate in the oceans is correlated with both the Great Oxidation Event (GOE) and the Proterozoic snowball glacial-interglacial cycles (Fru et al., 2015). The GOE involved a significant oxidation of the ocean-atmosphere system which both altered the balance between arsenite (As(III)) and arsenate (As(V)) but also triggered significantly increased chemical weathering of trace-metals from land to ocean (Canfield 1998; Reinhard et al., 2013; Fru et al., 2015). This enhanced weathering is believed to have brought with it a significant increase in the weathering of As-rich sulfide-minerals. Such exogenous weathering-associated input was punctuated by glacial-interglacial cycles and is thought to have resulted in oceanic As concentrations 3-4 fold higher than respective modern-day concentrations (Fru et al., 2015) (Figure 7). Molybdenum and vanadium are thought to follow a similar geological trajectory of efficient removal from the highly

sulfidic seawater characteristic of the Proterozoic (Javaux et al., 2001; Robbins et al., 2016) followed by increasing abundance upon ocean-atmosphere oxidation (Scott et al., 2008; Sahoo et al., 2012).

Phosphorus is typically considered to be the limiting nutrient for marine primary production on geological time scales (Tyrell, 1999). Although still an area of debate, a consensus is emerging of a significant change in marine P abundance, increasing to modern day concentrations from ~800 Ma. Suggesting that, throughout the Proterozoic and Archean, surface ocean P concentrations were persistently lower than modern-day equivalents (Jones et al., 2015; Reinhard et al., 2016) (Figure 7). The exact mechanism controlling marine P in Earth's Middle Ages, however, is not readily understood. Two dominant theories have been proposed i) elevated sorption of P to iron oxyhydroxides in an ocean rich in Fe^{2+} (Bjerrum and Canfield 2002) or ii) muted liberation of phosphorus during biomass remineralisation due to early oceans having a reduced oxidising capacity (Kipp et al., 2017). The chemical similarity of P, As, V and Mo mentioned previously would likely result in a broadly similar sorption mechanism. Therefore, decreases in P abundance would likely be mirrored in As, V and Mo. However, algal cellular stoichiometry of P is substantially higher than for either As, V or Mo. Therefore, if remineralisation, and so P liberation, were impeded by a lack of oxidising potential this would have a disproportionately negative effect on P relative to As, V or Mo. This disproportionality would allow for an environment with more significant

depletion of P relative to these other anions. Should such an environment have existed, our above-described mechanism of antagonistic co-limitation may have played a vital role in constraining early earth marine productivity.

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6. Conclusion

In this study, we present a comprehensive assessment of the role vanadate, arsenate and molybdate have on the diatom *T. oceanica*. For the case of vanadate and arsenate, we reveal the inhibitory effect that elevated concentrations of these compounds can have on growth rates, and demonstrate this effect being modulated by the algal population P sufficiency. We suggest this modulation is a consequence of ion promiscuity whereby chemically similar compounds are inadvertently taken up by seemingly poorly selective P uptake pathways. Interpreting these results under a framework of nutrient co-limitation allows us to demonstrate a novel scenario by which the limiting potential of one compound is dependent on the nutritional status of another - a phenomenon we term dependent antagonistic co-limitation. Additionally, we show significant accumulation of Vanadate and Arsenate during P limited growth, where we suggest this accumulation is unintentional and serves little if any biological purpose. A homeostatic trait, such as this, may go some way towards explaining some previously unexplained literature observations. These include the cellular accumulation of V (Tovar-Sanchez and Sanudo-Wilhelmy 2011; Nuester et al., 2011; Snow et al., 2014), elevated V content of some oil deposits (Treibs, 1936; Erickson et al., 1954; Vine and Tourtelot 1970), and the observed correlation of surface water V concentration with parameters more easily attributed to the resident

phytoplankton community of the North Atlantic (Klein et al., 2013; Pinedo-Gonzalez et al., 2015).

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Conflict of Interest

The authors declare no conflict of interest.

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Figure and Table Captions

Table 1: Total metal addition concentrations used in the study.

Concentration best representing environmental concentration of said metal is denoted with an asterisk and concentrations used for intracellular accumulation are denoted in bold.

Figure 1: Distribution and concentration of chemical species in media amendments at corresponding metal addition level. Concentrations of phosphate in P replete (purple) and P deplete (blue) conditions are shown as horizontal bars, separated by speciation HPO_4^{2-} (dashed), MgHPO_4 (solid) and NaHPO_4^- (dotted) which together account for >85% of the P added.

Figure 2: Maximum growth rate observed for each culture. Phosphate replete cultures are shown in purple and phosphate deplete cultures are shown in blue. Error bars represent the standard deviation of the mean.

Figure 3: Observed maximum growth rates (μ_{Max}) for P deplete large volume cultures. Metal addition (As = 20 nM, Mo = 100 nM and V = 500 nM) and no addition cultures are denoted with (+) or (-). As- and V- refer to the same physical culture where no V or As was added but Mo was present at 100 nM. *. Statistical significance was assessed by Student's t-test ($P > 0.05$, $n=3$).

Figure 4: Intracellular concentration of phosphorous, A) arsenic, B) molybdenum and C) vanadium under P deplete (blue) and P replete (purple). Error bars denote standard deviation and asterisks denote statistically significant differences on a per analyte basis (one-way ANOVA $P < 0.05$, $n=3$).

Figure 5: A) Intracellular concentration of P (purple) and V (orange) for differing P and V concentrations as determined from per cell V/P abundance and cell volume calculated from observed cell diameter, error bars represent standard deviation of the mean. B) P (purple) or V(orange) uptake and cellular enrichment relative to the media concentration (i.e. $[X]_{IC}/[X]_{Media}$).

Figure 6: Conceptual diagram showing potential scenarios of growth rate or yield stimulation through nutrient addition relative to a control population (C, black). Independent nutrient additions are shown for two nutrients (R_1 , yellow and R_2 , blue) alongside the sum of the individual effects (S, blue and yellow stacked) and the observed effect of addition of both nutrients (R_{1+2} , green). Population responses are shown for each of the 5 scenarios outlined in the text. Dashed line illustrates control growth rate for reference. Figure inspired by and modified from (Allgeier et al., 2011).

Figure 7: Schematic diagram depicting geological abundance of As (red), Mo (green), V (yellow) and P in surface oceans relative to modern day concentrations. Atmospheric oxygen is shaded in blue. Sources are As (Fru et al., 2015), Mo (Robbins et al., 2016), V (Sahoo et al., 2016).

Table 1: Total metal addition concentrations used in the study.

Concentration best representing environmental concentration of said metal is denoted with an asterisk and concentrations used for intracellular accumulation are denoted in bold.

| Metal (chemical species) | Concentration added (nM) |
|--|---|
| Vanadium (Na_3VO_4) | 0, 40*, 100, 250, 500, 750, 1,000, 10,000 |
| Arsenic (Na_3AsO_4) | 0, 20*, 100, 250, 500, 750, 1,000, 10,000 |
| Molybdenum ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) | 0, 25, 100*, 250, 500, 750, 1,000, 10,000 |

Graphical abstract

Highlights:

- Intracellular accumulation of V, As and Mo by phytoplankton are largely unexplained.
- Phosphorus limitation is common in the marine environment.
- Phosphate stress is exacerbated by the presence of P analogs: vanadate and arsenate.
- V/As accumulation maybe indicative of P limitation in the surface ocean.

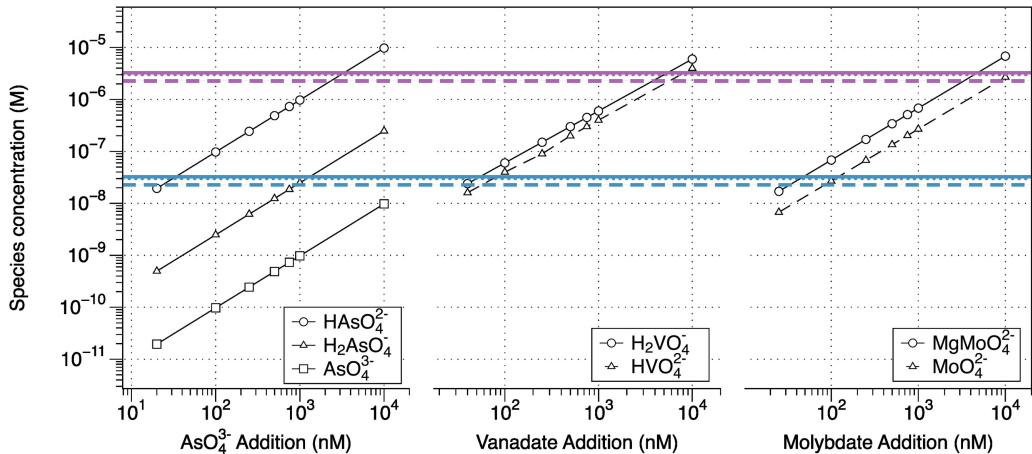


Figure 1

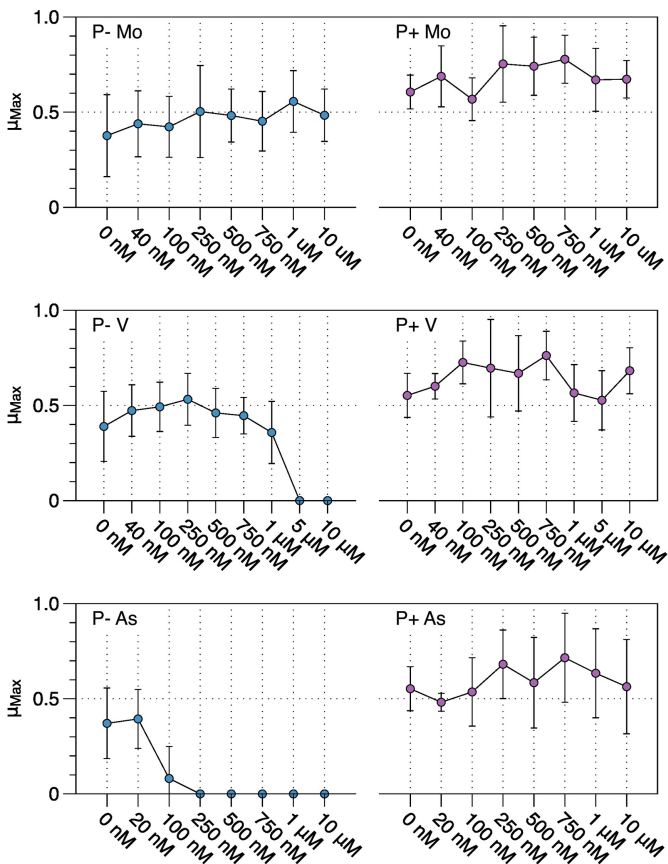


Figure 2

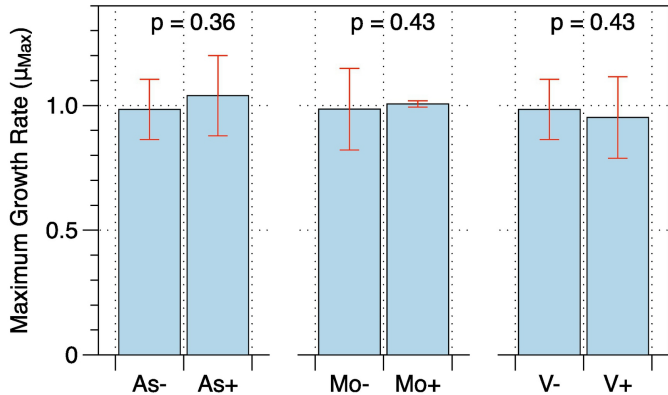


Figure 3

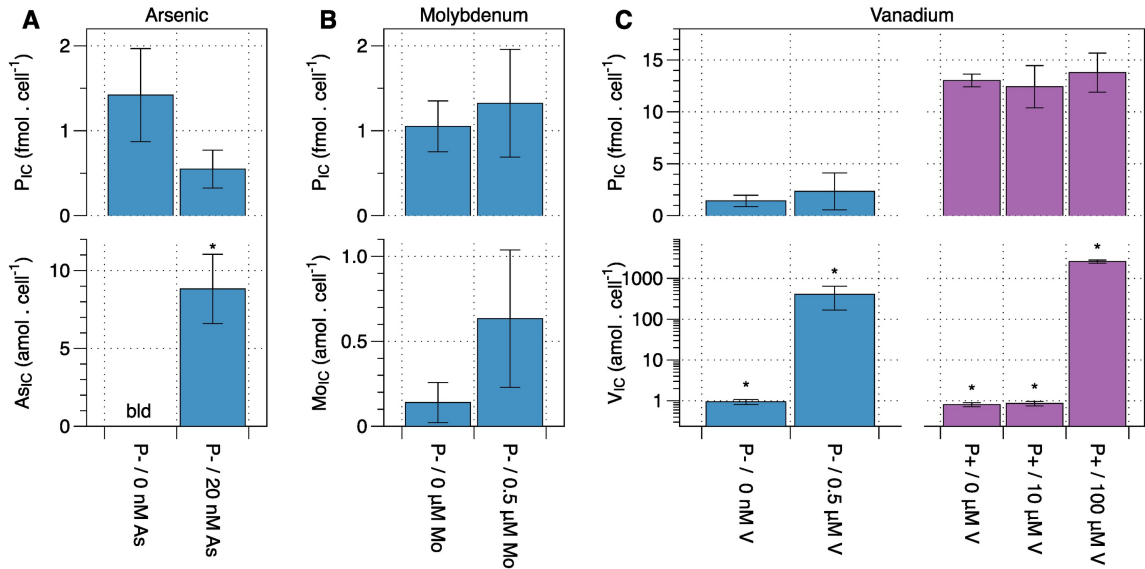


Figure 4

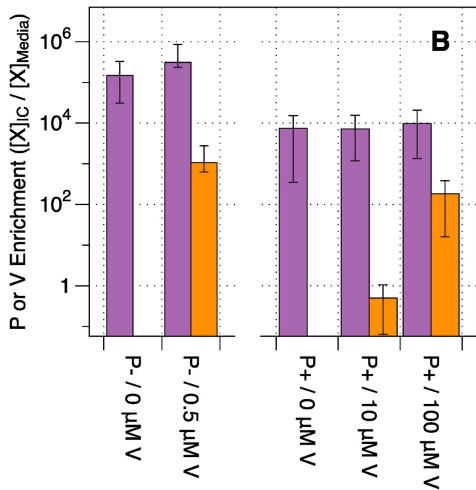
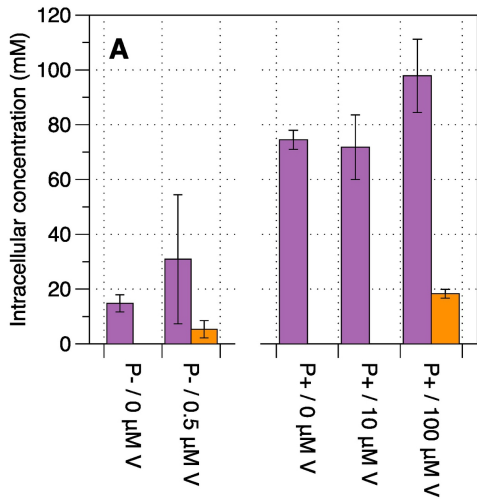


Figure 5

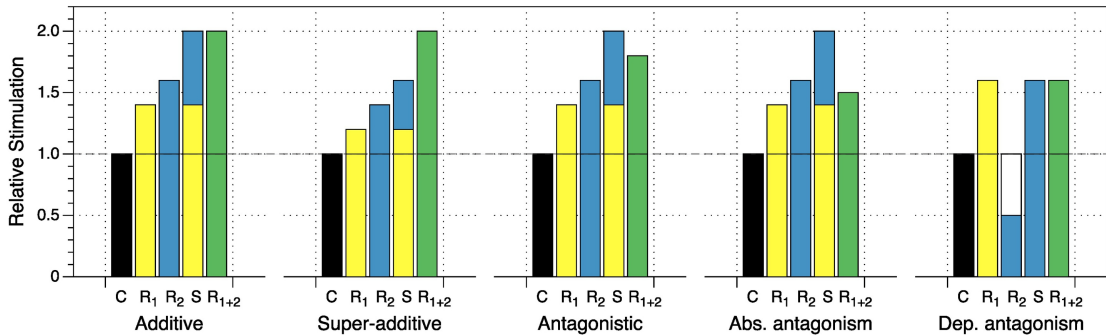


Figure 6

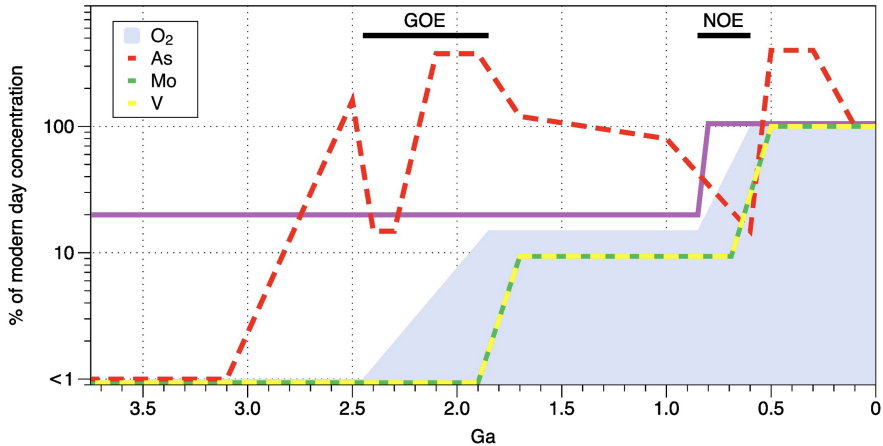


Figure 7