1 Supporting information for:

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3 Energy cost of intracellular metal and metalloid detoxification in wild-type eukaryotic phytoplankton

4 Michel Lavoie¹*, John A. Raven²⁻³, Oliver A. H. Jones⁴, Haifeng Qian⁵

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7 ¹Québec-Océan and Unité Mixte Internationale Takuvik Ulaval-CNRS, Département de Biologie, Université Laval, Québec, Québec, Canada G1K 7P4.
8 ²Division of Plant Science, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK
9 ³Functional Plant Biology and Climate Change Cluster (C3), University of Technology Sydney, Ultimo, NSW 2007, Australia
10 ⁴Australian Centre for Research on Separation Science (ACROSS), School of Science, RMIT University, GPO Box 2476, Melbourne, VIC 3001, Australia
11 ⁵College of Environment, Zhejiang University of Technology, Hangzhou 310032, P.R. of China
12
13 *corresponding author
14 Email: Michel_lavoie91@yahoo.ca
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1. Energy costs for biosynthesis of the amino acids that make up phytochelatins in algae

1.1 Energy cost of glutamate synthesis

Glutamate is predominantly produced in plant chloroplasts via the GS/GOGAT (glutamine synthetase/glutamate 2-oxoglutarate aminotransferase) cycle. This requires an N source (NO$_3^-$ or NH$_4^+$) and C assimilation into 2-oxoglutarate. Only a low fraction of glutamate is typically produced from the glutamate dehydrogenase pathway (GDH), that directly converts 1 mol 2-oxoglutarate to 1 mol glutamate using 1 mol NADH, and the GDH that often catalyzes the reverse reaction leading to deamination of glutamate to 2-oxoglutarate in plants.

In the GS/GOGAT cycle, 1 mol glutamate and 1 mol NH$_4^+$ are first converted to glutamine by the enzyme glutamine synthetase (GS) using 1 mol ATP. In all photosynthetic eukaryotes and cyanobacteria the ferredoxin-dependent enzyme glutamate synthase (also frequently called the GOGAT enzyme) converts glutamine in 2 glutamate using 2-oxoglutarate and 2 reducing equivalents as reduced ferredoxin (equivalent to 1 mol NAD(P)H). Glutamate can then re-enter the cycle or be used for biosynthesis. With an appropriate supply of 2-oxoglutarate and NH$_4^+$ (either taken up from the medium or reduced from NO$_3^-$), this cycle leads to the net synthesis of 1 mol glutamate. The
biochemical pathways and energetics of N uptake and assimilation as well as of 2-oxoglutarate biosynthesis are described below.

The energy required for the uptake of exogenous inorganic nitrogen and reduction (if needed) to NH$_4^+$, which is used in the GS/GOGAT cycle, is as follows. If NH$_4^+$ is the exogenous N source, 1 mol ATP is converted to 1 mol ADP for the uptake of 1 mol NH$_4^+$ coupled to 1 mol H$^+$ efflux by the H$^+$-ATPase plasma membrane pump. If NO$_3^-$ is the exogenous N source however, 1 mol ATP is first required per mol NO$_3^-$ taken up. NO$_3^-$ uptake occurs in algae as a secondary-active co-transport process in which H$^+$ efflux by the H$^+$-ATPase maintains the negative electrical membrane potential and indirectly energizes NO$_3^-$:H$^+$ or NO$_3^-$:Na$^+$ co-transport. Secondly, a total of 4 mol NAD(P)H (or their redox equivalent) are required for NO$_3^-$ reduction to NH$_4^+$, i.e. 1 mol NAD(P)H for reduction of 1 mol NO$_3^-$ and 6 reducing equivalents from reduced ferredoxin for the NO$_2^-$ to NH$_4^+$ reaction are required, this is equivalent to 3 mol NAD(P)H. Note that NH$_4^+$ can also be produced endogenously during the breakdown of storage proteins and the deamination of proteins and the catabolism of nucleic acids, but breakdown and regeneration of proteins for cell maintenance cannot sustain the addition of nitrogen in newly synthesized metal-binding peptides when nitrogen of proteins is recycled.

The other substrate of the GS/GOGAT cycle is 2-oxoglutarate. This molecule is produced via a complex set of enzymatic reactions. Briefly, the carbon fixed by the PCRC (Photosynthetic carbon reduction cycle or Benson-Calvin cycle) in the light is oxidized to pyruvate in the glycolysis pathway. Pyruvate is then converted to acetyl CoA, which is
used in the Krebs cycle to produce citrate, which is finally converted to 2-oxoglutarate by a sequence of two enzymatic reactions\textsuperscript{11}. The synthesis of 1 mol citrate involves the reaction of 1 mol acetyl CoA with 1 mol oxaloacetate. The removal of 1 mol 2-oxoglutarate to produce 1 mol glutamate means that oxaloacetate is not regenerated by the subsequent Krebs cycle reactions consuming 2-oxoglutarate, and continued production of 2-oxoglutarate requires synthesis of oxaloacetate from photosynthate\textsuperscript{11}. The energy cost of synthesis of 2 mol oxaloacetate is thus calculated below to account for the regeneration of this intermediate in the Krebs cycle as well as to its direct use in 2-oxoglutarate synthesis.

To produce the 5-C compound 2-oxoglutarate, 6 mol CO\textsubscript{2} must first be fixed to produce 2 mol 3-phosphoglycerate by RuBisCO and then be reduced to form 2 mol glyceraldehyde-3-phosphate (G-3-P) in the PCRC in the light. Note that G-3-P can also be generated from stored polysaccharide and then glycolysis, in the dark, although at an extra energy cost related to synthesising and then mobilising the polysaccharide. The minimum absorbed photon cost per C assimilated into 1/3 mol G-3-P at CO\textsubscript{2} saturation is 9 and costs 2 mol NADPH and 3 mol ATP\textsuperscript{12}. Thus, for synthesizing 2 mol G-3-P the minimum theoretical cost is \textbf{12 mol NADPH} and \textbf{18 mol ATP} and the absorbed photon cost is \textbf{54}. However, at present atmospheric CO\textsubscript{2} partial pressure or mol fraction (400 μmol CO\textsubscript{2} mol\textsuperscript{-1} total gases) the minimum absorbed photon cost per mol C fixed into 1/3 mol G-3-P for the least costly mechanism based on diffusive CO\textsubscript{2} entry and PCOC (Photosynthetic carbon oxidation cycle or photorespiration) activity is 9.96 (see the Methods section of the main text body), while the various CCMs have absorbed photons costs per mol C of
For 2 mol G-3-P the absorbed photon cost considering CCMs and PCOC operation is $57 - 66 \text{ mol} \times (9.5 \times 6 - 11 \times 6)$, i.e. less than 22% higher than the minimum theoretical photon cost of 54.

1 mol G-3-P (out of the 2 mol produced by the PCRC, see previous paragraph) is exported to the cytosol by a triose-P translocator (TPT) or antiporter that exchanges one mol triose P for 1 mol inorganic phosphate at no ATP or NAD(P)H cost. This 1 mol G-3-P in the cytosol is then converted into 1 mol pyruvate through glycolysis generating 1 mol NADH and 2 mol ATP. Pyruvate then enters the mitochondrion by a pyruvate transport protein mediating the electroneutral transport of pyruvate, which is driven by the differences in pH across mitochondrial membranes. 1 mol Pyruvate is converted to 1 mol acetyl CoA (with the production of 1 mol CO$_2$ and 1 mol NADH) that is combined with 1 mol oxaloacetate to produce 1 mol citrate. The citrate is then converted, via isocitrate, to 1 mol 2-oxoglutarate (with production of 1 mol CO$_2$ and 1 mol NADH).

Alternatively, pyruvate can be converted to citrate in the Krebs cycle in the mitochondrion, exiting via a DTC and then being converted in 2-oxoglutarate in the cytosol by aconitase and isocitrate dehydrogenase.

The other mol of G-3-P is converted to phosphoenolpyruvate (PEP) with production of 1 mol NADH and 1 mol ATP; the PEP is then converted to oxaloacetate using PEP carboxylase with consumption of 1 CO$_2$ (as HCO$_3^-$). This oxaloacetate regenerates the oxaloacetate used for 2-oxoglutarate synthesis (see previous paragraph). Oxaloacetate enters the mitochondrion, probably using an electroneutral dicarboxylate-tricarboxylate...
carrier (DTC) where it is used in producing citrate and properly fueling the Krebs cycle. Overall, 2 mol G-3-P are converted to 1 mol 2-oxoglutarate with net production of 1 mol CO₂, 4 mol NADH and 3 mol ATP.

Next, 2-oxoglutarate is pumped out of the mitochondrion through electroneutral dicarboxylate-tricarboxylate carriers (DTCs) that exchanges carboxylates at no ATP or NAD(P)H cost. Finally, the 2-oxoglutarate is transferred from the cytosol to the chloroplast for the GS/GOGAT cycle using a 2-oxoglutarate/malate exchanger at no ATP or NAD(P)H cost. Translocation of 2-oxoglutarate from the mitochondrion to the cytosol and the chloroplast does not change the energy cost (as NADH and ATP) of 2-oxoglutarate synthesis. Therefore, as stated above, 2 mol G-3-P are converted to 1 mol 2-oxoglutarate with net production of 1 mol CO₂, 4 mol NADH and 3 mol ATP.

Given the above, using NH₄⁺ as the N source synthesis of 1 mol glutamate from 2 mol G-3-P costs 2 mol ATP and 1 mol NAD(P)H (from the GS/GOGAT cycle and NH₄⁺ uptake) but generates 4 mol NADH and 3 mol ATP, equivalent to the net production of 3 mol NAD(P)H and 1 mol ATP. Using NO₃⁻ as the N source, synthesis of 1 mol glutamate from G-3-P costs 2 mol ATP and 5 mol NAD(P)H (from the GS/GOGAT cycle and NO₃⁻ reduction, see above), but generates 4 mol NADH and 3 mol ATP, equivalent to a net cost of 1 mol NAD(P)H with production of 1 mol ATP. Including the cost of synthesis of 2 mol G-3-P, i.e. 12 mol NAD(P)H and 18 mol ATP, the biosynthetic cost of 1 mol glutamate from N, CO₂, photosynthetically active radiation (PAR) and water is 9 mol.
NAD(P)H and 17 mol ATP if NH$_4^+$ is the N source or 13 mol NAD(P)H and 17 mol ATP if NO$_3^-$ is the N source.

Conversion of these values to photon requirements uses the value of 9 absorbed mol photons to produce 2 mol NADPH and 3 mol ATP in non-cyclic electron flow, which is equivalent to 4.5 absorbed mol photons per 1 mol NAD(P)H and 1.5 mol ATP. In cyclic electron flow, 1 absorbed mol photon is required to produce 1 mol ATP $^{12, 19}$. The synthesis of one mol glutamate from CO$_2$, PAR, water, and NH$_4^+$ as the N source requires 9 mol NAD(P)H, involving the coupled production of 13.5 mol ATP from non-cyclic electron flow, which is equivalent to (9 x 4.5) 40.5 mol absorbed photons. Since 17 mol ATP are required to synthesise 1 mol glutamate with NH$_4^+$ as N source, the additional (17-13.5) 3.5 mol ATP using cyclic electron flow costs 3.5 mol absorbed photons, i.e a total of (40.5+3.5) or 44 mol absorbed photons. If NO$_3^-$ is the N source, production of the 13 mol NAD(P)H and the coupled 19.5 mol ATP requires (13 x 4.5) 58.5 mol absorbed photons. The 19.5 mol ATP synthesised in non-cyclic electron transport is 2.5 mol ATP more than is needed in glutamate synthesis; the use of this 2.5 mol ATP in other essential processes spares the use of 2.5 mol absorbed photons, so the net photon requirement is (58.5-2.5) or 56 mol absorbed photons.

1.2 Energy cost of cysteine synthesis

The biosynthesis of cysteine requires SO$_4^{2-}$, CO$_2$, PAR, H$_2$O and a N source (NO$_3^-$ or NH$_4^+$). We start by considering the energetics of SO$_4^{2-}$ uptake and reduction. Sulphate...
uptake in plants across the plasmalemma uses ~1 mol ATP per mol SO\(_4^{2-}\) for a

3H\(^+\):1SO\(_4^{2-}\) symport \(^{20}\). Sulfate reduction to sulfide (S\(^2-\)) consumes 732 kJ mol\(^{-1}\), or 1 mol ATP, 6 electrons (usually from reduced ferredoxin, equivalent to 3 mol NAD(P)H) and 2 other electrons of GSH (equivalent to 1 mol NAD(P)H) \(^{21}\). Sulfate is first activated by ATP-sulfurylase in the presence of 1 mol ATP to form adenosine 5’-phosphosulphate (APS). One mol APS is then converted to 1 mol S-sulfoglutathione and 1 mol AMP by the enzyme APS sulfotransferase coupled to the oxidation of 1 mol GSH in GSSG and AMP plus PPI, equivalent to converting 2 mol ATP to ADP. Reduction of APS to SO\(_3^{2-}\) and AMP using 2 GSH converted to 1 GSSG; 1 NADPH is converted to NADP\(^+\) re-reducing the GSSG back to 2 GSH \(^{20,21}\). One mol SO\(_3^{2-}\) is reduced to 1 mol S\(^2-\) using 6 mol of reducing equivalents as reduced ferredoxin; this is equivalent to converting 3 mol NADPH to 3 mol NADP\(^+\). The S\(^2-\) then converts serine (see next paragraphs for details on the biochemical pathways leading to serine synthesis and on the energetics of serine synthesis) to cysteine using serine acetyl transferase and O-acetylserine thiol lyase (also called cysteine synthase). The use of acetyl CoA as a substrate and the production of CoA and acetate involve the conversion of 2 mol ATP to ADP and 2Pi in regeneration of 1 mol of the acetyl CoA by acetyl CoA synthetase. The energy required for the reduction of 1 mol SO\(_4^{2-}\) into S\(^2-\) and the production of 1 mol cysteine from serine and S\(^2-\) is 8 mol NAD(P)H and 7 mol ATP.

The serine, which is required as a substrate of the reactions leading to cysteine synthesis, is produced via CO\(_2\) fixation and assimilation in G-3-P by the PCRC followed by enzymatic conversion of G-3-P in serine. Although the PCOC cycle could synthesize...
serine, this cycle only functions in the light and with fluxes restricted due to the usual presence of a CCM in algae as explained in the section about glutamate synthesis. One mol of G-3-P can be first produced by the PCRC at a cost of 6 mol NADPH and 9 mol ATP (or 27 mol absorbed photons).

Afterwards, 1 mol G-3-P is converted to 1 mol serine using the following biochemical pathways. Conversion of 1 mol G-3-P to 1 mol 3-phosphoglycerate, via 1 mol glycerate-1,3-bisphosphate, converts 1 mol NAD(P)\(^+\) to 1 mol NAD(P)H, and 1 mol ADP to 1 mol ATP. Oxidation of 1 mol 3-phosphoglycerate to 3-phosphohydroxypyruvate involves reduction of 1 mol NAD(P)\(^+\) to 1 mol NAD(P)H. Amination of 2.5 mol (3-phospho)hydroxypyruvate to produce 1 mol 3-phosphoserine, using the GS/GOGAT pathway, consumes 1 mol NH\(_4^+\), and converts 1 mol ATP to 1 ADP and 1 mol NADH to 1 mol NAD\(^+\). If NH\(_4^+\) is the exogenous N source, 1 mol ATP is converted to 1 mol ADP for the uptake of 1 mol NH\(_4^+\) and the efflux of 1 mol H\(^+\), while if NO\(_3^-\) is the exogenous N source, 1 mol ATP is converted to 1 mol ADP for the uptake of 1 mol NO\(_3^-\) and 4 mol NAD(P)H are converted to 4 mol NAD(P)\(^+\) in reducing 1 mol NO\(_3^-\) to 1 mol NH\(_4^+\) (see previous section on glutamate synthesis for further details on NO\(_3^-\) reduction). For the conversion of 1 mol G-3-P into 1 mol serine, the balance of reduction-oxidation (NAD(P)H-NAD(P)\(^+\)) and dehydration-hydration (ATP + H\(_2\)O - ADP + Pi) for NH\(_4^+\) as N source is a production of 1 mol NAD(P)H and consumption of 1 mol ATP, while for NO\(_3^-\) as N source is a consumption of 3 mol NAD(P)H and of 1 mol ATP.
The net energy cost for the synthesis of 1 mol serine from CO$_2$, PAR and N is 5 mol NAD(P)H and 10 mol ATP using NH$_4^+$ as the N source or 9 mol NAD(P)H and 10 mol ATP using NO$_3^-$ as the N source, while the energy required for sulfate reduction and the production of 1 mol cysteine from S$_2^-$ and serine is 8 mol NAD(P)H and 7 mol ATP.

Finally, the total energy cost for the synthesis of one cysteine from CO$_2$, PAR, N, and SO$_4^{2-}$ is 13 mol NAD(P)H and 17 mol ATP if NH$_4^+$ is the N source or 17 mol NAD(P)H and 17 mol ATP if NO$_3^-$ is the N source.

Conversion of these values to photon requirements uses the values of 9 absorbed mol photons to produce 2 mol NADPH and 3 mol ATP in non-cyclic electron flow, which is equivalent to 4.5 absorbed mol photons per 1 mol NADPH and 1.5 mol ATP. In cyclic electron flow, 1 absorbed mol photon is required to produce 1 mol ATP. For the synthesis of 1 mol cysteine with NH$_4^+$ as the N source, production of 8 mol NADPH and 12 (8 x 1.5) mol ATP from non-cyclic electron flow requires 36 (8 x 4.5) mol absorbed photons. The remaining 3 mol ATPs can be produced by cyclic electron flow using 3 mol absorbed photons. For the synthesis of 1 mol cysteine with NO$_3^-$ as the N source, production of 13 mol NADPH and the coupled 19.5 mol ATP (13 x 1.5) from non-cyclic electron flow requires (13 x 4.5) 58.5 mol absorbed photons. Production of the 4.5 mol ATP in excess of that required for cysteine synthesis costs 4.5 mol absorbed photons by cyclic electron flow. The total energy cost for the synthesis of 1 mol cysteine is 39 and 54 mol absorbed photons if NH$_4^+$ and NO$_3^-$ are the N source respectively. The photon energy cost for the synthesis of one γ-EC unit is 83 (44 + 39) or 110 (56 + 54) mol absorbed photons if NH$_4^+$ and NO$_3^-$ is the N source, respectively.
1.3 Energy cost of glycine synthesis

Glycine can be produced by the glycolate cycle (PCOC or photorespiration or photorespiratory carbon oxidation cycle), but the PCOC only functions in the light and with fluxes restricted due to the usual presence of a CCM in algae as explained above. Therefore, glycine is expected to come from the PCRC products and glycolytic pathway intermediate, G-3-P. As detailed above, the PCRC and glycolysis produce serine. Serine and tetrahydrofolate (THF) are then converted in glycine and 5,10-methylenetetrahydrofolate (5, 10-CH$_2$-THF) by the enzyme serine hydroxymethylase. 5,10-CH$_2$-MTHF can be subsequently reduced to 5-methyltetrahydrofolate (5-CH$_3$-THF) by a 5,10-methylenetetrahydrofolate reductase enzyme that is NADH dependent in plants, not NADPH-dependent as in other eukaryotes. Finally, THF is recycled with methionine synthase using 5-CH$_3$-THF and homocysteine as substrates with no ATP or NAD(P)H inputs. Taking the energy cost of serine biosynthesis calculated in the previous section, the biosynthesis of 1 mol glycine from CO$_2$, PAR, and N requires 6 mol NAD(P)H and 10 mol ATP if NH$_4^+$ is the N source or 10 mol NAD(P)H and 10 mol ATP if NO$_3^-$ is the N source.

If NH$_4^+$ is used as an N source for the synthesis of 1 mol glycine, the production of 6 mol NADPH by non-cyclic electron flow and the associated 9 mol ATP requires 27 mol absorbed photons. 1 mol ATP can also be produced via cyclic-electron flow at a cost of 1 mol absorbed photons. If NO$_3^-$ is used as an N source for the synthesis of 1 mol glycine,
the production of 10 mol NADPH by non-cyclic electron flow and the associated 15 mol ATP requires 45 mol absorbed photons. The 5 mol ATP in excess would cost 5 mol absorbed photons, which needs to be subtracted of the 45 mol absorbed photons. The total energy cost for the synthesis of 1 mol glycine from CO₂, N, PAR and water is \(28\) and \(41\) mol absorbed photons if \(\text{NH}_4^+\) and \(\text{NO}_3^-\) are the N source respectively.

Therefore, the energy cost for the synthesis of 1 mol GSH is \(97\) (\(30 + 39 + 28\)) or \(125\) (\(30 + 54 + 41\)) mol absorbed photons if \(\text{NH}_4^+\) and \(\text{NO}_3^-\) is the N source, respectively.

\[2. \text{ Loss of peptides to the external medium and the energy cost of phytochelatin production}\]

Loss of GSH, amino acids and PCs in the external medium is not accounted for in the total energy cost of PC synthesis for the following reasons. Even though an appreciable fraction of the total intracellular GSH in \(\text{Thalassiosira weissflogii}\) exposed to highly inhibitory Cu (\(\mu\) of around 0.2 d\(^{-1}\)) may be lost into the culture medium each day probably because of Cu-induced cell breakage, the GSH loss rate in \(T. \text{weissflogii}\) is normally low (<10% of GSH cell quotas) at slightly toxic Cu concentration \(^{25}\). Similarly, exudation of GSH and cysteine in \(E. \text{huxleyi}\) only slightly increased in response to slightly toxic Cu, Cd or Zn concentrations and the amount lost each day likely remains a small fraction (<10%) of the total intracellular thiols \(^{26}\). Our calculation of the biosynthetic cost of PC also neglects active efflux of Me-PC complexes through a putative ABC membrane transporter. The occurrence of such an efflux of Cd-PC complexes has been strongly suggested in the marine diatom \(T. \text{weissflogii}\) \(^{27}\). Although this efflux system allow the fast efflux of Cd taken up (two ions are released in the
culture medium for each 4 Cd ion taken up), the efflux of phytochelatins (as Cd-PC complexes) accounted for around 10% of total steady-state intracellular phytochelatin produced each day by the alga 27.

3. Energy cost of Metal-PC complex transport in the vacuole and subsequent incorporation of sulfides

Metal-phytochelatins (and Metal-GSH) complexes (often called low molecular weight, or LMW, complexes) once formed in the cytosol can be transported into the vacuoles of fungi and plants by specific ATP-dependent transporters requiring 1 mol ATP per mol Metal-PC complex transported in the vacuole 28. Although the specific transporters of Metal-PC complexes are yet to be studied in algae, accumulation of Cd-PC complexes in the vacuole of Duniaella bioculata exposed to Cd has been observed by X-ray microscopy 29 and co-sequestration of Cd, N and S sulfur compounds has also been observed in the vacuole of Skeletonema costatum exposed to Cd and Cu 30. In plants, yeasts and algae, sulfide (S²⁻) is incorporated in some Cd-PC (or Cd-GSH) complexes forming a Cd sulfide crystalline core coated with PC. Such complexes called HMW Cd-PC complexes exhibit a high molecular weight (10 000 Da), are more stable and have a higher Cd binding capacity than LMW Cd-PC complexes. Whether or not HMW complexes are formed with other metal ions than Cd remains to be demonstrated 28. In LMW Cd-PC complexes, the Cys:Cd ratios (or SH:Cd) are often around 2:1 and 4:1 in yeast and algae 31-34, but can reach values near 0.6:1 in HMW Cd-PC complexes in P. tricornutum 32. Although synthesis of the LMW Cd-PC complexes are rapid occurring on a time scales of minutes 35, detectable conversion of LMW Cd-PC complexes into HMW
Cd-PC complexes in *P. tricornutum* and *C. reinhardtii* is slower and typically occurs after days of exposure. The HMW complexes dominate over the LMW-complexes after several days of Cd exposure, only when the cells grown in batch cultures are probably in the stationary growth phase. The S\(^{2-}:Cd\) ratio of the HMW Cd-PC complexes has been shown to be 0.4 in *P. tricornutum* and 0.22 in *C. reinhardtii*.

The source of the sulfide and the biosynthetic pathway of HMW complexes are unknown in algae and not fully known in yeast. In yeast, the limited scientific evidence available so far suggests that cysteine provides the sulfide of the HMW complexes according to the following reactions. First, cysteine sulfinate (CS) is derived from cysteine by cysteine dioxygenase. Second, CS is incorporated into either sulfinylpropanylaminomidazocarboxamide ribonucleotide (SPACR) or sulfinyl propanyl adenylate (SPA) by succinaminoimidazocarboxamide ribonucleotide synthetase (SAICAR synthetase) or adenylosuccinate synthetase (AMP-S synthetase) respectively. Both reaction requires the equivalent of 1 mol ATP since the SAICAR synthetase enzyme converts 1 ATP in 1 ADP and the AMP-S synthetase enzyme converts 1 GTP in 1 GDP. Third, both S analogs (SPACR and SPA) are presumably either donors or carriers of S\(^{2-}\) to Cd-GSH or PC-Cd complexes to form HMW GSH-CdS and PC-CdS complexes in the vacuole of algae and yeast. Note that either the GSH-Cd complex is transported in the vacuole and then converted in PC-Cd complexes with a vacuolar PCS or the PC-Cd complexes are directly transported in the vacuole; both translocations from the cytosol to the vacuole by a putative algal ABC transporters could cost ~1 ATP as previously observed in yeast. Note also that additional Cd\(^{2+}\) ions
could also be transported directly in the vacuole by specific transporters of Cd\(^{2+}\) ions in the tonoplast and contribute to the formation of HMW Cd-PC complex as proposed in yeast. In yeast, Cd\(^{2+}\) ion can be translocated from the cytosol to the vacuole by specific Cd\(^{2+}\) transporters using the proton gradient of vacuolar membrane and thus Cd\(^{2+}\):2H\(^+\) antiport. This H\(^+\) gradient is built by a H\(^+\)-ATPase located in plant and yeast tonoplast and 2 ATP are thus necessary to maintain the proton gradient for each Cd\(^{2+}\) ions accumulated in the vacuole. The stoichiometry of this reaction is however unknown in any organism, to our knowledge, and was not considered in our calculation of the energy cost of PC synthesis. Since the energy required for Cd\(^{2+}\) transport in the vacuole is a low fraction of the total energy cost of PC biosynthesis, this particular reaction could be neglected.

Overall, biosynthesis of HMW Cd-PC complexes costs 2 ATP per transfer of S\(^{2-}\) from cysteine to LMW Cd-PC complexes, while the production cost of 1 mol S\(^{2-}\) is 1 mol ATP and 4 mol NAD(P)H. Therefore, for the HMW Cd-PC complexes studied in *P. tricornutum* with a SH:Cd ratio of 0.6:1 and a S\(^{2-}\):Cd ratio of 0.4:1, which is equivalent to a SH:S\(^{2-}\) ratio of 1.5 or a S\(^{2-}\):SH ratio of 2/3, then the approximate cost of S\(^{2-}\) synthesis and incorporation in the HMW Cd-PC complex per SH of PCs is 2/3 x [3 ATP + 4 NAD(P)H], or 2 mol ATP and 8/3 mol NAD(P)H. The synthesis of 8/3 mol NADPH and the coupled 4 mol ATP by non-cyclic electron transport costs 12 mol absorbed photons. Subtracting the photon cost of the 2 mol ATP in excess (2 mol absorbed photons by cyclic electron transport), then the biosynthetic cost of S\(^{2-}\) and its incorporation in the HMW Cd-PC complex per 1 mol SH of PCs is 10 mol absorbed photons. This cost is low.
compared to the photon energy cost of the synthesis of 1 mol PCs in LMW Cd-PC complex, i.e. either $28 + 83n + 4n$ ($\text{NH}_4^+$ as the N source) or $41 + 110n + 4n$ ($\text{NO}_3^-$ as N source) mol absorbed photons (where n is expressed as mol SH groups per mol PCn) (see main text body).
4. Concentrations of phytochelatins in marine and freshwater phytoplankton exposed to Cd

Here we report intracellular phytochelatin concentrations measured in various marine and freshwater phytoplankton species exposed to Cd, the more potent inducer of phytochelatin. We consider PCs production in marine phytoplankton and then freshwater phytoplankton.

Rijstenbil and Wijnholds show that total cellular phytochelatin concentrations in four marine phytoplankton species (Ditylum brightwellii, Phaeodactylum tricornutum, Skeletonema costatum, and Thalassiosira pseudonana) reached up to 1.5 mmol SH of PCs per L\textsubscript{cell} after a 24 h-exposure to total Cu or Cd concentrations in the µM range, which significantly decreased cell viability. The marine coccolithophore E. huxleyi was the species synthesizing the highest PC concentration at the highest tested Cd concentration among 8 marine phytoplankton species examined by Ahner et al. At 1 nM free Cd\textsuperscript{2+}, a high concentration for marine environments but a Cd concentration around 4 times lower than the Cd toxicity threshold on the growth of E. huxleyi CCMP 374, E. huxleyi synthesize around 16 mmol SH of PC\textsubscript{n} (around 160 amol of PC\textsubscript{2} and PC\textsubscript{3} and assuming a cell volume of 50 µm\textsuperscript{3} as measured in control cells). Note, however, that the computation of intracellular PC concentration in E. huxleyi using the cell volume of control cell could lead to over-estimation of intracellular PC concentrations since Cd is
known to induce enlargement of algal cell size even at non-growth inhibitory
congeners.

The freshwater alga *C. reinhardtii* exposed for 72 h to 200 nM Cd\(^{2+}\) (around EC50 on
cell yield) synthesizes around 300 amol \(\gamma\)-GC unit (PC\(_2\) to PC\(_6\)) per cell. If the biovolume
is around 65 \(\mu\)m\(^3\) (control cells) but around 520 \(\mu\)m\(^3\) for Cd-intoxicated cells, then the
PCs concentration will be around 570 \(\mu\)mol EC units of PC per L\(_{\text{cell}}\) in Cd-exposed cells
\(33\). By comparison, the freshwater alga *Scenedesmus vacuolatus* exposed to the highest
Cd\(^{2+}\) concentration tested by Le Faucheur et al. \(44\), i.e. 79 nM Cd\(^{2+}\) (a concentration
inhibiting cell growth rate), synthesized around 200 amol SH of PCs per cell (mostly PC\(_2\)
and PC\(_3\)). Taking the cell volume of control cells (117 \(\mu\)m\(^3\)), which was similar to the cell
volume measured in cells exposed to 79 nM Cd\(^{2+}\), *S. vacuolatus* synthesized up to 1.7
mmol SH of PC per L\(_{\text{cell}}\).

A wild-type strain of the freshwater alga *Chlorella* sp. exposed for 4 days to 10 \(\mu\)M total
Cd (a concentration that strongly inhibits the growth rate by 70% relative to that of the
control) has been shown to synthesize 0.082 mmol PC (as GSH equivalent) per gram
fresh weight \(45\). Assuming a wet weight:carbon mass ratio of 10 \(46\), PC cell content is
equivalent to 8.2 \(\mu\)mol PC (as SH) per g carbon or 98.4 \(\mu\)mol PC per mol carbon.

Assuming an organic carbon cell concentration of 23.23 mol C L\(_{\text{cell}}^{-1}\) in freshwater algal
cells (see method section of the main text body), the intracellular PC concentration in that
wild-type strain of *Chlorella* is around 2.29 mmol SH of PCs per L\(_{\text{cell}}\). The study of
Kaplan et al. \(45\) also looked at the level of PCs synthesis in a resistant strain of *Chlorella*
sp. (CdR-DK) isolated from an urban waste water treatment plant. This strain constitutively synthesizes large amount of PCs, i.e. 2.165 mmol PC (as SH) per g fresh weight, which is equivalent to 60.4 mmol PC (as SH) per L_{cell} using the same assumptions as above. After 4 days of exposure to 100 µM total Cd (a Cd concentration inhibiting the growth rate by around 70%), the resistant strain of Chlorella sp. synthesizes 3.77 mmol PC (as SH) per g fresh weight or 105.2 mmol PC (as SH) per L_{cell}.

A Cr-tolerant and a wild-type strain of the freshwater green alga Scenedesmus acutus have been shown to accumulate up to around 8 µmol SH-PC per g dry weight after a 24 h exposure to total Cd concentrations of 2.25 to 4.5 µM (4.5 µM total Cd totally suppressed algal growth for the first 24 h) 47. Using a dry weight per cell biovolume of 498.5 g dry weight per liter determined in Scenedesmus quadriricauda 48, a strain closely related to S. acutus, S. acutus synthesize an approximate PCs concentration of 3.9 mmol PC-SH per L_{cell}.

5. Polyphosphate production in phytoplankton and energetics of polyphosphate synthesis and degradation

Polyphosphate (PolyP) concentrations in P-replete marine and freshwater phytoplankton cells usually represent a minor fraction of cell phosphorus (≤ 10% of total cellular P) 49-52. However, some algae such as the halotolerant algae Dunaliella salina can store large amount of PolyP of the order of 0.5 to 1 mol L^{-1}_{cell} so that cell phosphorus is mostly present as PolyP 53. The pavlovophyean (Haptophyta) Pavlova lutheri also stored large amount of PolyP (up to 70% of total cell phosphorus) when grown in the presence of
sufficient phosphate concentrations for growth. *Chlorella ellipsoidea* is another example of microalgae containing more than 10% of cell phosphorus in PolyP. Indeed, fractionation of intracellular P in this species performed by Miyachi and Tamiya has shown that PolyP constitutes around 31% of total cell P.

Polyphosphate can be synthesized via the two biochemical reactions and associated enzymes:

1) Polyphosphate kinase, which catalyzes the formation of orthophosphate chains from ATP by this reaction: \( \text{ATP} + (\text{phosphate})_n \rightleftharpoons \text{ADP} + (\text{phosphate})_{n+1} \); 

2) 3-phospho-glyceroyl-phosphate-polyphosphate phosphotransferase or bisphosphoglycerate-polyphosphate phosphotransferase, which catalyzes the reaction: \( 1,3\text{-bisphosphoglycerate} + (\text{phosphate})_n \rightleftharpoons 3\text{-phosphoglycerate} + (\text{phosphate})_{n+1} \)

In the first and the second reaction, the phosphorylation of the growing PolyP chain requires 1 mol ATP per mol phosphate added. In algae. However, in the second reaction, one may hypothesize that 1 mol 1,3-bisphosphoglycerate could be regenerated from 1 mol 3-phosphoglycerate by phosphoglycerate kinase at the expense of 1 mol ATP. The total cost (as ATP) of the second reaction is thus 2 mol ATP per mol phosphate group added to the PolyP chain. Note that throughout this study, we assume that PolyP is synthesized using the first scheme, i.e. the most parsimonious biochemical pathway.
Elongation of the PolyP chains requires phosphate uptake from the external medium. This phosphate group is taken up from the external medium either directly via membrane-bound orthophosphate transporters \(^{55-57}\) or indirectly from a range of other phosphate-containing molecules (e.g. nucleotides, glycerophosphates, polyphosphates) with extracellular phosphatase (alkaline phosphatase or nucleotidase) that cleaves phosphate \(^{57,58}\). Direct transport of phosphates in algal cells occurred either by active transport or facilitated diffusion ultimately requiring ATP \(^{57}\). In terrestrial plants, the uptake of 1 mol phosphate occurs via an H\(^+\) cotransporter (secondary active transport) and thus requires the efflux of 1 mol H\(^+\) by the ATPase at a cost of 1 mol ATP to maintain the transmembrane potential \(^{59}\). The same energy costs occur if Na\(^+\) replaces H\(^+\) in the phosphate transporter and the primary active, ATP-dependent, cation efflux \(^{60}\).

Cleavage of phosphate performed by the enzyme alkaline phosphatase and nucleotidase do not require ATP or NAD(P)H (Brenda website). Assuming that PolyP is produced via a polyphosphate kinase (via the most parsimonious reaction 1), the total cost of PolyP biosynthesis (as ATP) can thus be approximated to 2 mol ATP per mol PO\(_4^{3-}\) added to the polyphosphate chain.

Polyphosphate chains can also be degraded in orthophosphates by different transferases, kinases and hydrolases in bacteria and fungi \(^{61}\). The reaction catalysed by a polyphosphate kinase (see reaction 1 above) that produced polyphosphates can also run backward producing ATP or GTP and PO\(_4^{3-}\). AMP phosphotransferase can also catalyze the attack at a polyphosphate chain end by AMP producing ADP and Pi. As opposed to polyphosphate kinase and AMP-phosphotransferase, glucokinase requires energy as ATP.
to liberate orthophosphate from polyphosphate; glucokinase catalyzes the conversion of glucose in glucose-6-phosphates coupled to the conversion of 1 ATP in ADP. Finally, hydrolases or phosphatase cleave polyphosphate chains in the presence of water without the use of ATP or NAD(P)H.

Using a Redfield atomic ratio of 106:1 C:P, the total organic cell carbon concentrations in freshwater (23.23 mol C L\text{cell}^{-1}) or marine (11 mol C L\text{cell}^{-1}) algal cells, the energy cost of PolyP synthesis (2 mol ATP per mol PolyP or 2 mol absorbed photons per mol PolyP if ATP is produced via cyclic electron flow) and even assuming that virtually all intracellular P is incorporated in PolyP, we calculate that the cost of polyphosphate granules synthesis is only around 0.08% of the total biosynthetic cost of a freshwater or a marine cell.

6. Examples for some metals and algae species where no metal efflux was observed

In two freshwater green algae species (Pseudokirchneriella subcapitata and Chlorella kessleri), the studies of Hassler and Wilkinson and Wolterbeek et al. have suggested that zinc efflux of a fixed proportion of intracellular zinc occurred constitutively at low rates (efflux rates are less than 10% of Zn uptake rates) even at high toxic free Zn\text{2+} concentrations suggesting that Zn efflux is not a major inducible Zn detoxification mechanism in these algae species. In addition, Cd efflux of Cd-loaded P. subcapitata cells has been shown to be negligible over a 4-h period and modelling of Cd uptake in the freshwater alga C. reinhardtii exposed to a free Cd\text{2+} concentration of 7 nM (toxic on
cell division, but not yet toxic on cell volume yield) for 60 h suggests that Cd efflux is negligible\textsuperscript{67}. Moreover, Angel et al\textsuperscript{68} did not observe any Cu efflux by \textit{P. tricornutum} cells previously exposed to 15 $\mu$g L\textsuperscript{-1} total dissolved Cu for 72 h (a Cu concentration and exposure time inhibiting cell biomass by more than 70% relative to that of the control).

7. Energy cost of As reduction and methylation

7.1 Biochemical pathway and energy cost of As reduction and methylation

The biochemical pathway leading to methylation and reduction of As is poorly known in algae. Most of the knowledge we have of this pathway comes from the work performed in vertebrates and fungi (Hughes 2002, Bentley and Chasteen, 2002). Briefly, inorganic arsenate [iAs(V)] is thought to be reduced to inorganic arsenite [iAs(III)] followed by successive oxidative methylation and reduction reactions, which produce sequentially monomethylarsonic acid or MMA(V), monomethylarsonous acid or MMA(III), dimethylarsinic acid or DMA(V), dimethylarsinous acid or DMA(III) and trimethylarsenic oxide or TMAO(V), which is reduced to trimethylarsine gas or TMA(III) (reaction 3). This section describes the biochemical pathway and energy cost of As reduction and methylation.

\begin{align*}
\text{iAs(V)} + 2 \text{e}^- & \rightarrow \text{iAs(III)} + \text{CH}_3^+ \\
\text{MMA(V)} + 2 \text{e}^- & \rightarrow \text{MMA(III)} + \text{CH}_3^+ \\
\text{DMA(V)} + 2 \text{e}^- & \rightarrow \text{DMA(III)} + \text{CH}_3^+ \\
\text{TMAO(V)} + 2 \text{e}^- & \rightarrow \text{TMA(III)} \\
\end{align*}

(3)
Once in the cell, iAs(V) can be reduced to iAs(III) coupled to the oxidation of two GSH molecules. The regeneration of these 2 GSH via glutathione reductase cost 2 NADPH. Subsequently, iAs(III) could be complexed to GSH and/or exported out of the cells perhaps via silicic acid transporters (Lsi2), acting in silicic acid efflux with higher cytosol than external silicic acid concentration, as demonstrated in rice. However, the gene homolog coding for Lsi2 is not present in eukaryotic algae (http://www.ncbi.nlm.nih.gov). iAs(III) can also be reduced with GSH and can be methylated with As methyltransferases as shown in thermophilic and acidophilic algae. Note that this enzyme has been found in several algae species (http://www.ncbi.nlm.nih.gov). The methyl group used by As methyltransferase could be transferred by the S-adenosylmethionine (SAM or AdoMet) cycle. In this cycle, 5-methyltetrahydrofolate (N5-Me-THF) is used as a methyl donor in the SAM cycle and can be regenerated from 5N,10C methylene tetrahydrofolate (5N,10C methylene THF). 5N,10C methylene THF can be synthesized via two pathways at the same energy cost: 1) the serine-glycine cycle and 2) synthesis of serine from triose phosphate, conversion of serine to glycine and glycine catabolism. Here we start by computing the cost of 5N,10C methylene THF synthesis by both pathways before calculating the cost of the methyl transfer reactions in the SAM cycle.

The first possibility considered is that the 5N,10C methylene THF used in the synthesis of N5-Me-THF is assumed to come from the serine-glycine cycle. To allow for 5N,10C methylene THF synthesis in the dark requires that the starting point for the cycle is taken as serine derived from glycolytic or photosynthetic triose phosphate in glycolysis. The
serine-glycine cycle is initiated by the synthesis of 2 mol triose phosphate in
photosynthesis, which requires 6 x 2 or 12 mol NADPH and 9 x 2 or 18 mol ATP.
Conversion of 2 mol triose phosphate to 2 mol 3-phosphoglycerate produces 2 mol NADH
and 2 mol ATP. 2 mol 3-phosphoglycerate are converted to 2 mol glycerate by a
phosphatase and the 2 mol glycerate are converted to 2 mol hydroxypyruvate with
conversion of 2 mol NADH. Conversion of 2 mol hydroxypyruvate to 2 mol serine using
external NH$_4^+$ and the GS-GOGAT pathway requires 4 mol ATP and 2 mol NAD(P)H.
Overall, the production of 2 mol serine from 6 mol H$_2$O, 6 mol CO$_2$ and 2 external mol
NH$_4^+$ costs 10 mol NAD(P)H and 20 mol ATP. Conversion of 2 mol serine to 2 mol
glycine converts 2 mol THF to 2 mol 5N,10C methylene THF. Conversion of 2 mol
glycine to 1 mol serine involves production of 1 mol CO$_2$, 1 mol NH$_4^+$ and 1 mol NADH.
This initial reaction sequence leading to the synthesis of 1 mol serine converts 6 mol
CO$_2$, 6 mol H$_2$O, 2 external mol NH$_4^+$ and 2 mol THF to 1 mol serine, 3 mol CO$_2$, 1 mol
internal NH$_4^+$ and 2 mol 5N,10C methylene THF. The overall cost is 9 mol NAD(P)H
and 20 mol ATP with a cost of 40.5 mol absorbed photons to produce 9 mol NAD(P)H
and 13.5 mol ATP by non-cyclic electron flow, and 6.5 mol absorbed photons to produce
the remaining 6.5 mol ATP by cyclic electron flow, i.e. 47 mol absorbed photon to
convert 2 mol THF to 2 mol 5N,10C methylene THF, or 23.5 mol photons per 1 mol THF
converted to 1 mol 5N,10C methylene THF.

Subsequent to this, the serine-glycine cycle requires the synthesis of another molecule of
serine besides the serine from the initial round of conversion of 2 mol THF to 2 mol
5N,10C methylene THF. Synthesis of the additional serine, using internal NH$_4^+$ from the
conversion of 2 glycine to 1 serine in the initial round costs 5 mol NAD(P)H and 9 mol ATP per serine. Conversion of the 2 serine to 2 glycine, and then conversion of the 2 glycine to 1 serine, produces 2 mol 5N,10C methylene THF, 1 mol CO$_2$, 1 mol NADH and 1 mol internal NH$_4^+$.

This gives an overall cost of 4 mol NAD(P)H and 9 mol ATP in producing 2 mol 5N,10C methylene THF, i.e. 2 mol NAD(P)H and 4.5 mol ATP for the production of 1 mol 5N,10C methylene THF. The production of 2 mol NADPH and 3 mol ATP costs **9 mol absorbed photons** in non-cyclic electron flow, and production of the remaining 1.5 mol ATP by cyclic electron flow costs **1.5 absorbed photons**, a total of **10.5 mol absorbed photons**.

5N,10C methylene THF can also be synthesized from CO$_2$, PAR, NH$_4^+$ and water via a second pathway involving the production of serine from triose phosphate in glycolysis, the conversion of serine to glycine and the catabolism of glycine to CO$_2$, NH$_4^+$ and 2 molecules of 5N,10C methylene THF. Synthesis of 1 mol triose P in photosynthesis from H$_2$O and CO$_2$ uses 6 mol NADPH and 9 mol ATP. Conversion of 1 mol triose phosphate to 1 mol 3-phosphoglycerate produces 1 mol NADH and 1 mol ATP; phosphatase activity produces 1 mol glycerate from 1 mol 3-phosphoglycerate and this 1 mol glycerate is converted to 1 mol hydroxypyruvate with production of 1 mol NADH. Conversion of 1 mol hydroxypyruvate to 1 mol serine using external NH$_4^+$ and the GS-GOGAT pathway requires 2 mol ATP and 1 mol NAD(P)H. Overall, the production of 1 mol serine from H$_2$O, CO$_2$ and external NH$_4^+$ costs 5 mol NAD(P)H and 10 mol ATP. Conversion of 1 mol serine to 1 mol glycine converts 1 mol tetrahydrofolate (THF) to 1 mol 5N,10C methylene THF. Catabolism of 1 mol glycine to 1 mol CO$_2$, 1 mol NH$_4^+$ and 1 mol
NADH converts 1 mol THF to 1 mol 5N,10C methylene THF. Summing these costs, 4 mol NAD(P)H and 10 mol ATP convert 2 mol THF to 2 mol 5N,10C methylene THF. In the steady state of 5N,10C methylene THF synthesis the NH₄⁺ produced in glycine catabolism can be used in amination of hydroxypyruvate, thus saving the 1 ATP used in NH₄⁺ influx, so that the overall cost is 4 mol NAD(P)H and 9 mol ATP to convert 2 mol THF to 2 mol 5N,10C methylene THF. For converting 1 mol THF to 1 mol 5N,10C methylene THF the cost is 2 mol NAD(P)H and 4.5 mol ATP, equivalent to 10.5 mol NAD(P)H and 9 mol ATP. Overall, the SAM cycle, using 5N,10C methylene THF generated above and the precursors [arsenite, MMA(III), DMA(III)] to be methylated. In the SAM cycle, a methyl group (derived by reduction from 5N,10C methylene THF) is first transferred from SAM or AdoMet to a precursor [arsenite, MMA(III), DMA(III)] by a transferase (in this case, As methyltransferase), producing S-adenosylhomocysteine (SAH). Second, using the enzyme S-adenosylhomocysteine hydrolase, SAH is converted into adenosine and homocysteine in the presence of water and the enzyme cofactor (NAD⁺) is converted into NADH. Third, homocysteine is converted into methionine through transfer of a methyl group from 5-methyltetrahydrofolate (N5-Me-THF) by a methionine synthase (vitamin B12-dependent or -independent) producing tetrahydrofolate (THF). Four, N5-Me-THF is regenerated from 5N,10C methylene THF via the enzyme methylene tetrahydrofolate reductase, which uses 1 NADPH. Finally, 1 mol SAM is recycled from 1 mol methionine using the enzyme methionine adenosyltransferase, which uses 1 mol ATP.
which transfers a methyl group to As methyl transferase, requires 1 NADPH and 1 ATP, but also generates 1 NADH.

In summary, iAs(V) is first reduced to iAs(III) at a cost of 2 NAD(P)H for the regeneration of two GSH molecules. iAs(III) can then be sequentially reduced by GSH and methylated by the SAM cycle three times each yielding TMAO and TMA. The three reduction steps proceed at a potential cost of 6 NADPH if GSH reduces As(V) species and has to be regenerated. The mechanistic cost of reduction/methylation of 1 mol As(V) (without taking into account the biosynthetic cost of methyl groups) is 11 mol NAD(P)H and 3 mol ATP coupled to the production of 3 mol NADH, i.e. a cost of 8 mol NAD(P)H and 3 mol ATP. Adding the cost of 5,10 methylene THF synthesis for the three rounds of the SAM cycle (required to transfer three methyl groups to oxidised inorganic or organic As(III) species), the total energy cost of iAs(V) reduction and methylation from CO$_2$, PAR, and water is 14 mol NAD(P)H and 16.5 mol ATP.

Conversion of the ATP and NAD(P)H cost of As reduction and methylation to photon requirements uses the values of 9 absorbed mol photons to produce 2 mol NADPH and 3 mol ATP in non-cyclic electron flow, which is equivalent to 4.5 absorbed mol photons per 1 mol NADPH and 1.5 mol ATP. In cyclic electron flow, 1 absorbed mol photon is required to produce 1 mol ATP. The production of 14 mol NADPH (as well as the 21 mol coupled ATP) by non-cyclic electron flow required for the reduction/methylation of 1 mol As(V) costs 63 mol photons ($14 \times 4.5$). The 4.5 mol ATP in excess would have cost 4.5 mol absorbed photons if they had been produced via cyclic electron flow.
Consequently, the photon energy cost for the reduction/methylation of 1 mol As(V) is \(58.5\) mol absorbed photons.

### 7.2 Experimental measurements of As accumulation in phytoplankton

The intracellular As concentration that inhibits cell growth of eukaryotic algae species differs by several orders of magnitude depending mostly on the species. Indeed, we looked at six reports in the literature, in which As uptake has been measured in parallel to toxicity on cell growth and convert the amount of bioaccumulated As per cell dry weigh into cellular As concentration normalized to cell biovolume. Our aimed here was to explore the variability of As cell concentration in different phytoplankton species beyond which As toxicity effects on growth occurs in order to constrains a probable maximum As intracellular concentration and evaluate the probable upper bound energy cost of detoxification of such a high As cellular concentration.

We first consider the study of Knauer and Hemond. These authors studied As toxicity and accumulation in the green alga, *Chlorella sp.*, isolated from Upper Mystic Lake, a arsenic-contaminated lake near Boston, MA. At a concentration of 1 nM total As(V) and high P, which is already slightly toxic on the cell yield obtained after the exponential growth phase of 2 weeks, the As cell quotas (intracellular and adsorbed As; no phosphate rinsing steps were used to desorb As at the cell surface) are less than \(10^{-10}\) mol As (mg DW algae\(^{-1}\)) corresponding to an As accumulation rate less than \(10^{-4}\) \(\mu\)mol As per mg DW algae per day and As reduction rate less than \(10^{-11}\) mol As per mg DW algae per day.
According to Nalewajko \textsuperscript{48}, a strain of \textit{Chlorella} (\textit{Chlorella pyrenoidosa}) has 255 µg DW per µL of biovolume (or 255 mg DW per 10\textsuperscript{-3} L of biovolume). Converting the data of Knauer and Hemond \textsuperscript{73} on an “approximate” basis of cell biovolume instead of cell dry weight means that the As cell quotas are less than 2.6 x 10\textsuperscript{-5} mol As L\textsubscript{cell}\textsuperscript{-1} corresponding to an As accumulation rate less than 26 µmol As per L\textsubscript{cell}\textsuperscript{-1} per day and As reduction rate less than 2.55 µmol As per L\textsubscript{cell}\textsuperscript{-1} per day.

In the second study considered here, i.e. the paper of Maeda et al. \textsuperscript{74}, 67 mM inorganic As(V) started to decrease the cell yield (20% cell yield inhibition) of \textit{C. vulgaris} obtained after growing a culture up to the stationary growth phase at As cell quotas (only rinsed with water!) of around 18-19 mg As per g dry weight or 0.24-0.26 mmol As per g DW or 240 to 260 nmol As per mg DW. In this case, the cellular As concentration required to inhibit cell growth appears to be very high (around 58 to 62 mM assuming 255 µg DW per µL of biovolume \textsuperscript{48}) although these cell concentrations overestimate the true intracellular As concentration since the As adsorbed onto the cells was not removed in that study. Moreover, they only measured the cell yield in the stationary phase. Measuring As toxicity in the stationary growth phase could underestimate As toxicity if algae deplete dissolved As concentration in solution and then resumed their growth through As biodilution and then may reach higher final cell density in the stationary growth phase (relative to the case where dissolved As concentration is buffered in solution) after a longer log growth phase than the As-free control.
The third study was that of Karadjova et al. In that study, the As cellular quotas (rinsed with phosphate) (measured after 72-h of exposure to inorganic As(V) or As(III) ) close to the threshold of As toxicity on growth rate of the marine green alga *Chlorella salina* was around $10^{-18}$ mol As per cell at three different external phosphorus concentration (compared Fig. 2 and Fig. 3). In their Fig. 5A, the relative proportion of each As species (As(V), As(III), MMA, DMA, DHF) accumulated within the algae is shown. Taking a cell volume of *C. salina* of 116.29 µm$^3$ per cell, the intracellular As concenctration required to inhibit *C. salina* growth is around 8.6 µmol As per L-cell.

Fourth, Levy et al. calculated a LOEC for a 72-h exposure to As(III) of 3.75 mg L$^{-1}$ in *Monoraphidium arcuatum*. For As(V), the LOECs were 0.081 and 1.91 mg L$^{-1}$ for initial PO$_4^{3-}$ concentration of 0.15 and 1.5 mg L$^{-1}$ (i.e. molar N:P ratios of 150:1 and 15:1 respectively). At 125 µg initial As(V) L$^{-1}$ and 0.15 mg P L$^{-1}$ (i.e. an As(V) concentration close to the LOEC 72 h), the mean As cell quota (cells rinsed with phosphate) after 72 h was $1200 \times 10^{-18}$ g cell$^{-1}$ (16 $\times 10^{-18}$ mol cell$^{-1}$). At 1000 µg As(V) L$^{-1}$ and 1.5 mg L$^{-1}$ (close to the LOEC of 1.91 mg L$^{-1}$), mean As cell quota (cells rinsed with phosphate) after 72 h was $400 \times 10^{-18}$ g cell$^{-1}$ (5.3 $\times 10^{-18}$ mol cell$^{-1}$). Taking a relatively modest cell volume of 42 µm$^3$, the intracellular As concentration required to inhibit *M. arcuatum* growth is around 1.2 to 3.7 $\times 10^{-4}$ mol L$_{\text{cell}}^{-1}$.

Fifth, in the study of Pawlik-Skoronska et al., a 24-h exposure of the green microalga *Stichococcus bacillaris* to a slightly growth-inhibitory As(V) concentration (100 µM) at pH 6.8 and 8.2 resulted in As cell content of 4.04 and 1.27 µmol g$^{-1}$ DW, respectively.
Taking a value of the dry weight per cell biovolume of *S. bacillaris* of around 231.7 g DW per L of cell volume, we calculated that cellular concentrations of 295 to 936 µmol As per L<sub>cell</sub> are slightly toxic to *S. bacillaris*.

Sixth, Wang et al. have found that at As(V) concentrations slightly toxic to *Scenedesmus obliquus*, less than 10<sup>4</sup> µg As per g DW was accumulated inside the cells (the cells were rinsed with phosphate). For *Scenedesmus obliquus*, the EC50 on growth rate was 12929 µg intracellular As per g DW (or 172.6 µmol As per g DW) in medium with low P (no EC50 available at high P). For *Chlamydomonas reinhardtii*, the EC50 was 3298 and 408 µg As per g DW (or 44.0 and 5.45 µmol As per g DW) in the presence of high and low P, respectively (Table 1). Using values determined in *Chlamydomonas angulosa* of 317.8 g DW per L of algae and in *Scenedesmus quadricauda* of 498.5 g DW per L of algae, the EC50 on a cell volume basis can be estimated, i.e. an EC50 of 86 mmol As per L<sub>cell</sub> for *S. obliquus* and EC50s of 1.7 to 14 mmol As per L<sub>cell</sub> for *C. reinhardtii*.

7.3 Calculating the energy cost of As biotransformation in relation to As accumulation

It has been shown that up to around 30% of cellular As(V) can be reduced into As(III) and part of this As(III) is then methylated although at rates typically much lower than As(V) reduction rate and the methylated As species account for a low proportion of total cellular As. There is also a part of cellular As(III) that is excreted in solution.
This As efflux is thought to be relatively slow in *Chlorella salina*. Indeed, this species exposed to 10 µM As(V) for 72 h accumulated around $10^{-17}$ mol As per cell, but excrete in the dissolved phase 55 to 65% of the total cellular As over 72 h of depuration.

Assuming a specific growth rate of 1 d$^{-1}$ or a doubling time $[\ln(2)/\mu]$ of 0.693 d or 16.6 h and a constant As loss rate over the 72 h exposure, only 13 to 15% of cell As is lost during the period for growing a whole cell. Taking a mean cell volume of *C. salina* of 116 µm$^3$ per cell, the As cellular concentration lost during a period of one cell doubling is only around 11 to 13 µM As. Assuming an energy cost of 1 ATP per mol of As exported from the cells, based on the ATP cost for transport of numerous ions across the plasmalemma and considering a cost of 1 mol absorbed photons per mol ATP produced via cyclic electron flow, the energy cost of As(III) efflux is only 2.0 to 2.3 x $10^{-6}$ % of the total energy cost for growing a *C. salina* cell.

Considering that the proportion of total As cellular concentration detoxified by reduction and methylation is lower than 30% and that As efflux is slow, then, even with some allowance for the slow As efflux, the steady-state cellular concentration of detoxified As is expected to be lower than the total measured cellular steady-state As concentration. For algal cells accumulating at most around 50 mM As(V) concentrations at the onset of As toxicity on growth (20% cell yield inhibition after several days of growth), the energy cost invested in As detoxification is therefore probably lower than 2.95 mol absorbed photons per L$_{cell}$ (assuming that all cellular As is detoxified and using a cost of 58.5 mol absorbed photons per mol reduced/methylated As(V), see section 7.1), which is 0.5% of...
the total photon energy cost for growth of a freshwater cell (557.52 mol absorbed photons per L<sub>cell</sub>; see method section of the main text body).
8. Hg reduction: Biochemical mechanisms, occurrence in algae and energy cost

8.1 Biochemical mechanism of Hg reduction and energy cost of Hg reduction

Phytoplankton can reduced Hg(II) into Hg(0) as a detoxification mechanism. Although several cases of As methylation have been reported in the literature, no evidence currently exists, to our knowledge, supporting the methylation of Hg in phytoplankton.

The mechanism of Hg reduction in algae is still unknown, but studies have shown that the reduction depends on Hg concentration, but not light. Since plasmalemma redox enzymes reducing Cu(II) and Fe(III) exist in phytoplankton, one might hypothesize that Hg(II) is inadvertently reduced by such enzymes. The genes of a putative Fe reductase (Fre1p in yeast) have been found in marine diatoms suggesting that Fe reduction and uptake occurs in diatoms with similar pathways than that found in yeast. The Fre1p enzyme couples Fe(III) reduction into Fe(II) to NADPH oxidation.

Assuming that Hg reduction in phytoplankton proceeds via an unspecific reaction such as plasmalemma redox enzymes, which use NADPH, then the cost of Hg reduction into Hg(0) is only 1 mol NAD(P)H per mol Hg(II) reduced. Production of 1 mol NADPH costs 4.5 mol absorbed photons, but generates 1.5 mol ATP via non-cyclic electron flow. This ATP would have cost 1.5 mol absorbed photons when produced by cyclic electron
transport. Therefore, the net cost of unspecific Hg reduction is \textbf{3 mol absorbed photons per mol of reduced Hg(II)}. 

\textbf{8.2 Measurements of Hg reduction in the literature and Hg reduction energy cost relative to energy for cell growth}

The freshwater alga, \textit{Euglena gracilis}, has been shown to reduce Hg(II) at rates of 2.2 fmol Hg cell\(^{-1}\) h\(^{-1}\) and 0.7 fmol Hg cell\(^{-1}\) h\(^{-1}\) when exposed to a rather high Hg(II) concentration of 5 µM for 1 h and 3 h, respectively\(^\text{84}\). Using a cell volume of 22.3 pL\(^\text{91}\), the alga reduces Hg at rates of 31.4 to 98.6 µmol Hg L\(_{cell}\)\(^{-1}\) h\(^{-1}\). Taking a specific growth rate of around 1.2 d\(^{-1}\)\(^\text{92}\), equivalent to a doubling time of around 14 h, the amount of Hg reduced per unit of cell volume for the growth of a whole cell can reach around 1.4 mmol Hg per L\(_{cell}\).

The marine diatom, \textit{Thalassiosira weissflogii}, exposed to 5 nM Hg(II) in the light or in the dark produced around 92 zmol Hg(0) cell\(^{-1}\) h\(^{-1}\). This concentration of Hg(II) did not affect (or slightly affected by less than 10\%) the specific growth rate of this species. Based on a cell volume of 1370 µm\(^3\) or 1.37 pL\(^\text{93}\), it means that the Hg reduction rate of \textit{T. weissflogii} is around 67 nmol L\(_{cell}\)\(^{-1}\) h\(^{-1}\). Thus for a specific growth rate of 1 d\(^{-1}\)\(^\text{85}\) or a doubling time of 0.69 day (Ln(2)/1) or around 17 h, then the amount of cellular Hg that can be reduced during the growth of a cell is around 1 µmol L\(_{cell}\)\(^{-1}\).
Mason et al. have shown that the Hg production rate in a range of eukaryotic marine phytoplankton species (*Thalassiosira weissflogii*, *Dunaliella tertiolecta*, *Pavlova lutheri*, *Pleurochrysis carterae*) exposed to 0.5 nM Hg(II) vary between 1.1 and 20 zmol Hg cell\(^{-1}\) h\(^{-1}\). The Hg(II) concentration is probably not toxic on cell growth since the reported EC50 of Hg(II) in *T. weissflogii* and *D. tertiolecta* is 250 and 5000 nM Hg(II), respectively. These Hg reduction rates when converted on a biovolume basis vary between 1.7 to 234 nmol Hg L\(^{-1}\) cell\(^{-1}\) h\(^{-1}\), which is of the same order of magnitude than the Hg reduction rate measured by Morelli et al.

Wu and Wang found that Hg(0) production rate in three marine phytoplankton species (*Chlorella autotrophica*, *Thalassiosira weissflogii*, and *Isochrysis galbana*) exposed to 250 nM initial Hg(II) for 72 h was equal to or lower than around 0.06 pmol mol C\(^{-1}\) h\(^{-1}\) (their Fig 3B). Using a mean intracellular C concentration of 11 mol C per L\(_{\text{cell}}\) determined by Ho et al. in 15 marine phytoplankton species, the above Hg(0) production rate measured by Wu and Wang is equal to 0.66 pmol L\(_{\text{cell}}\)\(^{-1}\) h\(^{-1}\), which is much lower than the Hg reduction rates determined by Mason et al. and Morelli et al.

Therefore, at low nM Hg(II) exposure concentrations associated to the reduction of one micromole Hg per L\(_{\text{cell}}\) for the time required for a cell doubling, the photon cost for Hg reduction for the growth of a whole cell is expected to be on the range of micromole absorbed photons per L\(_{\text{cell}}\) (the cost of Hg reduction is around 3 mol absorbed photons per mol of reduced Hg(II)). Therefore, the net cost of Hg reduction represents a very low
proportion (~10^{-7} to 10^{-6} %) of cellular energy for the growth of a whole cell. For
Pavlova lutheri, the phytoplankton species investigated showing the fastest Hg reduction
rate (234 nmol Hg L_{cell}^{-1} h^{-1}), the energy cost associated to Hg reduction over the period
of a cell doubling (assuming μ≈ 1 d^{-1}; as measured by Thompson et al. 96) is only 5 x 10^{-6}
% of the total cellular energy cost. Even if the Hg reduction was as high as that measured
in E. gracilis exposed to 5 µM Hg (i.e. close to 100 µmol Hg L_{cell}^{-1} h^{-1}), the photon cost
of Hg reduction would still be a very low proportion of the total photon energy cost for
growth (8 x 10^{-4} %).

9. Energetics of the ascorbate-glutathione cycle, glutathione peroxidase cycle and
redox proteins

The hydrogen peroxide produced by the reaction of SOD with superoxide can be
detoxified to water by catalase, the ascorbate-glutathione cycle and the glutathione
peroxidase (GPX) cycle. Within the ascorbate-glutathione cycle, ascorbate peroxidase
(APX) first oxidizes ascorbate into monodehydroascorbate (MDA), which is reduces
back into ascorbate by MDA reductase (MDAR) at the cost of 1 NADPH. Additionally,
some MDA is spontaneously converted into dehydroascorbate (DHA), which can be
reduced to ascorbate by DHA reductase (DHAR) with the help of GSH that is oxidized to
GSSG. The GSH of the former reaction is regenerated by glutathione reductase (GR),
which re-converts GSSG in GSH. Alternatively, the GPX cycle converts H_{2}O_{2} into water
using reducing equivalents from GSH. GSH is again regenerated from GSSG with the
help of GR at a cost of 1 NADPH 97.
Apart from the classical enzymes (e.g. SOD, CAT, APX, and GR) protecting against oxidative stress, three other more recently identified redox proteins (thioredoxin, glutaredoxin and peroxiredoxin) in plants and algae are also involved in detoxifying ROS. The first redox proteins called thioredoxins can directly reduce $H_2O_2$, dehydroascorbate, and certain radicals in plant and algal cells. They can act as electron donors and thus facilitates the reduction of other proteins such as another redox protein, peroxiredoxin, that scavenges cellular hydroperoxides. The oxidized disulfide thioredoxins can be reduced back in thioredoxins by the flavoenzyme thioredoxin reductase in a NAD(P)H-dependent reaction. Second, glutaredoxins are redox proteins sharing many functions of thioredoxins, but that can be reduced by GSH. Glutaredoxins can reduce dehydroascorbate, $H_2O_2$ and APX. Since the reduced GSH used in the reaction of glutaredoxins can be regenerated with GR, both glutaredoxin and thioredoxin cost 1 NAD(P)H. Third, the thiol groups of peroxiredoxin reduces $H_2O_2$, hydroperoxides and peroxynitrites. The oxidized peroxiredoxin is then reduced with the help of thioredoxin or glutaredoxin regenerating the functional peroxiredoxin with reduced thiol groups.

Therefore, the energy cost to detoxify each ROS with the help of thioredoxin, glutaredoxin and peroxiredoxin is one NAD(P)H.

10. Hydrophilic nonenzymatic antioxidant

GSH is thought to have a pivotal role in metal(loid) detoxification due to metal(loid) complexation, its ROS detoxification ability and its high concentration in the mM range.
Steady-state total GSH concentration is usually tightly regulated in freshwater algae species at a fixed total concentration in response to low non-growth inhibitory Cd and Cu exposure \(^{33, 44, 99, 100}\). By contrast, GSH cellular concentration (amount of GSH per L\(_{\text{cell}}\)) is expected to decrease at higher toxic Cd concentrations on growth even though GSH cell quotas did not change significantly \(^{33, 44}\) since algal cell volume sensitively increase at [Cd] that inhibits the growth of \(C. reinhardtii\) \(^{101}\). Note that at high growth-inhibitory Cu concentrations, GSH concentrations decreased in \(C. vulgaris\) \(^{100}\). Also, exposure to the metalloids As (concentration inhibiting by 12 to 27% the growth rate) and Sb (non-inhibitory concentration) respectively caused an increase and a decrease in GSH cell quotas of \(Scenedesmus vacuolatus\), but potential changes in cell volume were not reported and calculation of GSH cell concentration cannot be performed \(^{41}\). Once GSH is converted in GSSG in the presence of ROS, GSH can be rapidly regenerated via glutathione reductase at a cost of only 1 NAD(P)H \(^{102}\). Taking a second order rate constant of GSH oxidation by the \(\cdot\)OH radicals \(^{103}\) of \(1.4 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}\) and assuming \(10^{-18}\) M \(\cdot\)OH in unstressed cells \(^{104}\), it follows that over the period of a cell doubling (17 h assuming a specific growth rate of 1 d\(^{-1}\)), less than 0.1% of total cellular GSH is oxidized by \(\cdot\)OH in unstressed cell. Regeneration of 0.1% of a cellular pool of 10 mM GSH costs only \(3 \times 10^{-5} \text{ mol absorbed photons}\), i.e., less than around \(10^{-5}\) % of the total energy cost for growth. Consequently, the energy cost of GSH regeneration (only considering GSH oxidation by the radical \(\cdot\)OH), particularly in algal cells exposed to metal(loid) concentrations close to the NOEC, is likely a low proportion of the total energy cost for growth.
Ascorbate is another major antioxidant in algal cells present at millimolar concentrations. Ascorbate concentrations in a range of fresh and marine phytoplankton species, which were compiled in 1995 by Raven \(^{105}\), were approximately between 0.4 and 28 mmol L\(_{\text{cell}}^{-1}\). Subsequent measurements by Urzica et al. \(^{106}\) of the mean intracellular ascorbate concentration in *C. reinhardtii* cells cultivated under optimal growth conditions yielded values around 0.1 mmol L\(_{\text{cell}}^{-1}\) \(^{106}\) using a critical cell volume of 140 fL at which synchronized *C. reinhardtii* cells (12 h ligh: 12h dark cycle) divide \(^{107}\). Also, Sunda et al. \(^{103}\) reported intracellular ascorbate concentrations of around 6 mM in *E. huxleyi*. Oxidized ascorbate can be reduced back into ascorbate by MDA reductase (MDAR) at the cost of only 1 NAD(P)H or 3 mol absorbed photons (i.e., 4.5 mol photons per mol NAD(P)H by non-cyclic electron flow minus the cost saved for synthesizing the coupled 1.5 mol ATP or 1.5 mol photons by cyclic electron flow). Taking a second order rate constant of ASC oxidation by the •OH radicals of 1.1 x 10\(^{10}\) M\(^{-1}\) s\(^{-1}\) tabulated in Sunda et al. \(^{103}\) and assuming 10\(^{-18}\) M •OH in unstressed cells \(^{104}\), it follows that over the period of a cell doubling (17 h assuming a specific growth rate of 1 d\(^{-1}\)), less than 0.1% of total cellular ASC is oxidized by •OH in unstressed cell. Regeneration of 0.1% of a cellular pool of 6 mM costs only 1.8 x 10\(^{-5}\) mol absorbed photons, i.e., less than 10\(^{-5}\) % of the total energy cost for growth. Consequently, the replacement cost of ASC (only considering ASC oxidation by •OH), particularly in algal cells exposed to metal(loid) concentrations close to the NOEC, is likely a low proportion of the total energy cost for growth.

The following reasoning further illustrates that regeneration of reduced ASC and GSH is likely a minor energy expenditure. Indeed, taking GSH and ASC cell concentrations of
10 mM and assuming that all the GSH and ASC pool is oxidized and enzymatically
regulated over the course of a cell doubling at a cost of 1 NAD(P)H (or 3 mol absorbed
photons) per mol ASC or GSH, this regeneration of ASC and GSH would each cost 3 x
10^{-2} mol absorbed photons, i.e., around 5.4 x 10^{-3} % and 1.1 x 10^{-2} % of the total energy
required for growing a whole freshwater or marine phytoplankton cell, respectively. This
clearly shows that even if the turnover of GSH and ASC in algal cells was rapid,
regeneration of both antioxidants would likely be a minor energy expenditure.

In plants, proline is mainly synthesized from glutamate in the cytosol with the help of two
enzymes. Glutamate is first reduced to glutamate-semialdehyde (GSA) by the pyrroline-
5-carboxylate synthetase (P5CS) enzyme. Second, GSA is spontaneously converted to
pyrroline-5-carboxylate (P5C). Third, the P5C intermediate is reduced to proline by the
P5C reductase (P5CR). Each enzymatic reduction requires 1 NAD(P)H. Therefore,
the biosynthesis of 1 mol proline from 1 mol glutamate costs 2 mol NAD(P)H while the
biosynthesis of 1 mol glutamate costs 9 mol NAD(P)H and 17 mol ATP if NH_{4}^{+} is the N
source or 13 mol NAD(P)H and 17 mol ATP if NO_{3}^{-} is the N source (See section on the
energy cost of phytochelatin synthesis). Therefore, the energy cost of biosynthesis of 1
mol proline from CO_{2}, N, PAR and water is 11 mol NAD(P)H and 17 mol ATP if NH_{4}^{+}
is the N source or 15 mol NAD(P)H and 17 mol ATP if NO_{3}^{-} is the N source. On a basis
of absorbed photons, the biosynthetic cost of 1 mol proline is either 50 or 62 mol
absorbed photons if NH_{4}^{+} and NO_{3}^{-}, respectively, are the N source.
The basal intracellular concentration of free proline in freshwater phytoplankton is in the range 100 µM to 3 mM, based on published measurements of free proline quotas in algae and conversion with cell volumes measured in each species. By comparison, internal proline concentrations in marine phytoplankton vary between 2 mM and 340 mM at the salinity of seawater (around 600 mM NaCl). Intracellular proline concentrations in freshwater phytoplankton were shown to increase in response to metal exposure. The intracellular proline concentration in *Chlorella vulgaris* was shown to increase from 0.8 to around 8 mmol L\(^{-1}\) after 10 h of exposure to 2.5 µM total Cu and 5 µM total Cr. These Cu and Cr treatments inhibited the algal growth rate by around 15% and 20% respectively. However, the cellular proline level returns to near basal levels after 25 h of exposure. The study of Mallick also showed that the cellular proline concentration in *Chlorella vulgaris* did not significantly increase for a 72-h exposure at low (non-growth-inhibitory) Cu concentrations (72 h), but at toxic Cu concentrations on growth, proline started to increase. In the species *Chlorella* sp., the intracellular free proline concentrations also increased in response to exposures to toxic Cu or Cd concentrations on growth, but the intracellular proline concentrations remained below around 290 µmol L\(^{-1}\). The observed transitory induction of proline biosynthesis at internal concentrations below 8 mM is expected to contribute a minor fraction (<0.1%) of the total energy cost for growth.

DMSP and GBT are effective antioxidant in marine phytoplankton although less effective scavenger of the •OH radical than ASC and GSH. DMSP and GBT can reach high concentrations usually on the order of 1-200 mM in marine diatoms and
coccolithophores \textsuperscript{119,120} although very high DMSP concentration up to around 1 M has been reported in free-living marine dinoflagellates \textsuperscript{121}. Since there are no known pathways that can effectively recycle DMSP and GBT (analogous to the recycling of GSSG in GSH by the GR), both antioxidants need to be \textit{de novo} synthesized at relatively high photon energy costs compare to those of other antioxidants. Indeed, \textit{de novo} steady-state biosynthesis of 1 mol DMSP in phytoplankton costs 93 mol absorbed photons whereas \textit{de novo} biosynthesis of 1 mol GBT costs 91 to 108 mol absorbed photons using NH$_4^+$ or NO$_3^-$ as the N source \textsuperscript{122}. Although DMSP and GBT could be a costly process for ROS scavenging if their synthesis were strongly up-regulated, no studies, to our knowledge, have shown an increase in DMSP or GBT concentrations in marine phytoplankton exposed to toxic concentrations of metal(loid)s. Indeed, DMSP intracellular concentrations are not up-regulated (and not totally regenerated) in the marine algae \textit{E. huxleyi} exposed to a growth-inhibitory Cu concentration (1 nM Cu$^{2+}$), but rather are decreased under Cu stress \textsuperscript{103}.

Even though turnover rates of DMSP in metal(loid)-exposed cells are currently unknown, we attempted to constrain the probable energy cost due to DMSP regeneration by \textit{de novo} DMSP biosynthesis. According to the following reasoning, significant depletion of intracellular DMSP due to oxidation by ROS (and significant energy loss due to DMSP oxidation) is unlikely at least at metal(loid) concentration close to the NOEC. Indeed, based on the published rate constant of DMSP oxidation by $\cdot$OH (k = 9 x 10$^8$ M$^{-1}$ s$^{-1}$) and $\cdot$O$_2^-$ (k = 8.3 M$^{-1}$ s$^{-1}$) radicals \textsuperscript{123}, an intracellular $\cdot$OH concentration in unstressed cells of 10$^{-18}$ M \textsuperscript{104} and a chloroplastic $\cdot$O$_2^-$ concentration of around 10$^{-9}$ M \textsuperscript{124}, less than 10$^{-4}$ % of
intracellular DMSP should be oxidized by these two radicals over a period of one day. This suggests that even if a considerable increase in ROS production under a severe metal(loid) stress occurred, the experimentally measured cellular DMSP pool would not decrease significantly. Even at the high intracellular DMSP concentrations measured in marine dinoflagellates (≈1 M) and assuming a doubling time of 17 h ($\mu = 1 \text{ d}^{-1}$), less than $10^{-6}$ M DMSP is expected to be consumed by reaction with •OH and •O$_2^-$. Assuming that the total cellular pool of DMSP is kept constant due to \textit{in vivo} DMSP production in control cells, then the photon cost of DMSP regeneration is $93 \times 10^{-6}$ mol absorbed photons, i.e., less than $10^{-2}$ % of the total photon energy cost for growth. Hence, even for a one or two orders of magnitude increase in •OH and •O$_2^-$ concentrations in cells exposed to metal(loid)s, regeneration of DMSP by \textit{de novo} DMSP synthesis would likely be a minor energy expenditure. Likewise, since GBT is a less effective hydroxyl radical scavenger than DMSP, the cost for maintaining the intracellular pool of GBT constant by \textit{de novo} GBT synthesis in conditions of oxidative stress is also expected to be minor.

11. Lipophilic non-enzymatic antioxidants

Intracellular concentrations of lipophilic carotenoids that specifically protect the chloroplast apparatus from ROS generally remain constant, or decrease, in microalgae exposed to Cd or Cu. Only in a few cases did carotenoids increase, but at growth-inhibitory metal concentrations, suggesting that they are not on the first line of defense against ROS in algae. Indeed, exposure of \textit{Scenedesmus} sp. to a toxic Cu concentration
(on O₂ evolution and cell viability) of 2.5 μM for 6 h or 7 days did not affect carotenoid cell quotas 125. Also, growth-inhibitory Cd concentrations did not affect the carotenoid content of the diatom *Nitschia palea* 126, 127, but strongly affect antioxidant enzymes and proline accumulation 127. Likewise, chronic metal stress (0.5 mg Cd L⁻¹ for 30 days) in the dinoflagellate *Gonyaulax polyedra* does not influence β-carotene (a carotenoid) synthesis but increased SOD and APX activity 128. It is known that a 24-h exposure to a high total Cd concentration (20 mg L⁻¹ in reconstitute seawater with no strong metal ligands added) inhibit epoxidation of diatoxanthin to diadinoxanthin, which are xanthophyll classified as carotenoids, in *Phaeodactylum tricornutum* 129. Furthermore, growth-inhibitory Cd concentrations decreased caroteinoid cell content in *Senedesmus bijugatus* 126. Only in the study of Mallick 116 and Bossuyt and Janssen 130 did the carotenoid cell content of *Chlorella vulgaris* and *P. subcapitata* increased in response to growth-inhibitory Cu concentrations (no evidence of carotenoid increase was found at non-growth inhibitory Cu concentrations in both studies).

Regarding the effects of metal(lloid)s on other lipophilic antioxidants such as tocopherols in microalgae, to the best of our knowledge, no studies have yet been performed on this topic.
12. References


76. J. Zeilinger, University of Vienna, 2011.


