Supporting information for:

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

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Table of contents

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 [1-3]. 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 [4-6], and the GDH that often catalyzes the reverse reaction leading to deamination of glutamate to 2-oxoglutarate in plants [1, 3].

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 ferrodoxin-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 [3]. 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 [7]. 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 [8, 9]. 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 [9, 10]. 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 [11], 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 [11]. 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 [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[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_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_2 saturation is 9 and costs 2 mol NADPH and 3 mol ATP [12]. Thus, for synthesizing 2 mol G-3-P the minimum theoretical cost is **12 mol NADPH** and **18 mol ATP** and the absorbed photon cost is **54**. However, at present atmospheric CO_2 partial pressure or mol fraction (400 μmol CO_2 mol^{-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_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

9.5 – 11 [12, 13]. For 2 mol G-3-P the absorbed photon cost considering CCMs and PCOC operation is 57 – 66 mol (9.5 x 6 – 11 x 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 [14]. 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 [15-17]. Overall, 2 mol G-3-P are converted to 1 mol 2-oxoglutarate with net production of 1 mol CO_2, 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 [18]. Finally, the 2-oxoglutarate is transferred from the cytosol to the chloroplast for the GS/GOGAT cycle using a 2-oxoglutarate/malate exchanger [11] 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 CO_2, 4 mol NADH and 3 mol ATP.

Given the above, using NH_4^+ 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 NH_4^+ 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 NO_3^- 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 NO_3^- 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, CO_2, 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_2O 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_2O - 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 [12, 19]. 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 cyle (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 [22]. 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 [23]. Finally, THF is recycled with methionine synthase using 5-CH_3-THF and homocysteine as substrates with no ATP or NAD(P)H inputs [24]. 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 CO_2, N, PAR and water is **28 and 41 mol absorbed photons** if NH_4^+ and 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 NH_4^+ and NO_3^- is the N source, respectively.

2. 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 *Thalassiosira weissflogii* exposed to highly inhibitory Cu (μ 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. weissflogii* is normally low (<10% of GSH cell quotas) at slightly toxic Cu concentration [25]. Similarly, exudation of GSH and cysteine in *E. 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. 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 *Dunialella 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 (S^{2-}) 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 [32, 36]. The S^{2-}:Cd ratio of the HMW Cd-PC complexes has been shown to be 0.4 in *P. tricornutum* [32] and 0.22 in *C. reinhardtii* [36].

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 sulfinylpropanylaminoimidazoecarboxamide ribonucleotide (SPACR) or sulfinyl propanyl adenylate (SPA) by succinoaminoimidazolecarboxamide 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 [37, 38]. 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 [28, 32]. 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 [28, 39]. Note also that additional Cd^{2+} ions

348 could also be transported directly in the vacuole by specific transporters of Cd^{2+} ions in
349 the tonoplast and contribute to the formation of HMW Cd-PC complex as proposed in
350 yeast [39]. In yeast, Cd^{2+} ion can be translocated from the cytosol to the vacuole by specific
351 Cd^{2+} transporters using the proton gradient of vacuolar membrane and thus Cd^{2+}:$2H^{+}$
352 antiport. This H^{+} gradient is built by a H^{+}-ATPase located in plant and yeast tonoplast
353 and 2 ATP are thus necessary to maintain the proton gradient for each Cd^{2+} ions
354 accumulated in the vacuole [39]. The stoichiometry of this reaction is however unknown in
355 any organism, to our knowledge, and was not considered in our calculation of the energy
356 cost of PC synthesis. Since the energy required for Cd^{2+} transport in the vacuole is a low
357 fraction of the total energy cost of PC biosynthesis, this particular reaction could be
358 neglected.
359
360 Overall, biosynthesis of HMW Cd-PC complexes costs 2 ATP per transfer of S^{2-} from
361 cysteine to LMW Cd-PC complexes, while the production cost of 1 mol S^{2-} is 1 mol ATP
362 and 4 mol NAD(P)H. Therefore, for the HMW Cd-PC complexes studied in *P.*
363 *tricornutum* [32] with a SH:Cd ratio of 0.6:1 and a S^{2-}:Cd ratio of 0.4:1, which is equivalent
364 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
365 and incorporation in the HMW Cd-PC complex per SH of PCs is 2/3 x [3 ATP + 4
366 NAD(P)H], or 2 mol ATP and 8/3 mol NAD(P)H. The synthesis of 8/3 mol NADPH and
367 the coupled 4 mol ATP by non-cyclic electron transport costs 12 mol absorbed photons.
368 Subtracting the photon cost of the 2 mol ATP in excess (2 mol absorbed photons by
369 cyclic electron transport), then the biosynthetic cost of S^{2-} and its incorporation in the
370 HMW Cd-PC complex per 1 mol SH of PCs is 10 mol absorbed photons. This cost is low

371 compared to the photon energy cost of the synthesis of 1 mol PCs in LMW Cd-PC
372 complex, i.e. either 28 + 83n +4n (NH_4^+ as the N source) or 41 + 110n + 4n (NO_3^- as N
373 source) mol absorbed photons (where n is expressed as mol SH groups per mol PCn) (see
374 main text body).
375

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 [28, 40, 41]. We consider PCs production in marine phytoplankton and then freshwater phytoplankton.

Rijstenbil and Wijnholds [42] 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_{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. [34]. At 1 nM free Cd^{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 [43], *E. huxleyi* synthesize around 16 mmol SH of PCn (around 160 amol of PC_2 and PC_3 and assuming a cell volume of 50 μm^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

398 known to induce enlargement of algal cell size even at non-growth inhibitory
399 concentrations.
400
401 The freshwater alga *C. reinhardtii* exposed for 72 h to 200 nM Cd^{2+} (around EC50 on
402 cell yield) synthesizes around 300 amol γ-GC unit (PC_2 to PC_6) per cell. If the biovolume
403 is around 65 μm^3 (control cells) but around 520 μm^3 for Cd-intoxicated cells, then the
404 PCs concentration will be around 570 µmol EC units of PC per L_{cell} in Cd-exposed cells
405 [33]. By comparison, the freshwater alga *Scenedesmus vacuolatus* exposed to the highest
406 Cd^{2+} concentration tested by Le Faucheur et al. [44], i.e.79 nM Cd^{2+} (a concentration
407 inhibiting cell growth rate), synthesized around 200 amol SH of PCs per cell (mostly PC_2
408 and PC_3). Taking the cell volume of control cells (117 μm^3), which was similar to the cell
409 volume measured in cells exposed to 79 nM Cd^{2+}, *S. vacuolatus* synthesized up to 1.7
410 mmol SH of PC per L_{cell}.
411
412 A wild-type strain of the freshwater alga *Chlorella* sp. exposed for 4 days to 10 µM total
413 Cd (a concentration that strongly inhibits the growth rate by 70% relative to that of the
414 control) has been shown to synthesize 0.082 mmol PC (as GSH equivalent) per gram
415 fresh weight [45]. Assuming a wet weight:carbon mass ratio of 10 [46], PC cell content is
416 equivalent to 8.2 µmol PC (as SH) per g carbon or 98.4 µmol PC per mol carbon.
417 Assuming an organic carbon cell concentration of 23.23 mol C L_{cell}^{-1} in freshwater algal
418 cells (see method section of the main text body), the intracellular PC concentration in that
419 wild-type strain of *Chlorella* is around 2.29 mmol SH of PCs per L_{cell}. The study of
420 Kaplan et al. [45] also looked at the level of PCs synthesis in a resistant strain of *Chlorella*

421 sp. (CdR-DK) isolated from an urban waste water treatment plant. This strain
422 constitutively synthesizes large amount of PCs, i.e. 2.165 mmol PC (as SH) per g fresh
423 weight, which is equivalent to 60.4 mmol PC (as SH) per L_{cell} using the same
424 assumptions as above. After 4 days of exposure to 100 µM total Cd (a Cd concentration
425 inhibiting the growth rate by around 70%), the resistant strain of *Chlorella* sp.
426 synthesizes 3.77 mmol PC (as SH) per g fresh weight or 105.2 mmol PC (as SH) per L_{cell}.
427 A Cr-tolerant and a wild-type strain of the freshwater green alga *Scenedesmus acutus*
428 have been shown to accumulate up to around 8 µmol SH-PC per g dry weight after a 24 h
429 exposure to total Cd concentrations of 2.25 to 4.5 µM (4.5 µM total Cd totally suppressed
430 algal growth for the first 24 h) [47]. Using a dry weight per cell biovolume of 498.5 g dry
431 weight per liter determined in *Scendesmus quadricauda* [48], a strain closely related to *S.*
432 *acutus*, *S. acutus* synthesize an approximate PCs concentration of 3.9 mmol PC-SH per
433 L_{cell}.
434
435 **5. Polyphosphate production in phytoplankton and energetics of polyphosphate**
436 **synthesis and degradation**
437

438 Polyphosphate (PolyP) concentrations in P-replete marine and freshwater phytoplankton
439 cells usually represent a minor fraction of cell phosphorus (≤ 10% of total cellular P) [49-52].
440 However, some algae such as the halotolerant algae *Dunaliella salina* can store large
441 amount of PolyP of the order of 0.5 to 1 mol L_{cell}^{-1} so that cell phosphorus is mostly
442 present as PolyP [53]. The pavlovophyean (Haptophyta) *Pavlova lutheri* also stored large
443 amount of PolyP (up to 70% of total cell phosphorus) when grown in the presence of

444 sufficient phosphate concentrations for growth [50]. *Chlorella ellipsoidea* is another
445 example of microalgae containing more than 10% of cell phosphorus in PolyP. Indeed,
446 fractionation of intracellular P in this species performed by Miyachi and Tamiya [54] has
447 shown that PolyP constitutes around 31% of total cell P.
448
449 Polyphosphate can be synthesized via the two biochemical reactions and associated
450 enzymes:
451
452 1) Polyphosphate kinase, which catalyzes the formation of orthophosphate chains
453 from ATP by this reaction: ATP + $(phosphate)_n \rightleftharpoons$ ADP + $(phosphate)_{n+1}$;
454 2) 3-phospho-glyceroyl-phosphate-polyphosphate phosphotransferase or
455 bisphosphoglycerate-polyphosphate phosphotransferase, which catalyzes the
456 reaction: 1,3-bisphosphoglycerate + (phosphate)n $\rightleftharpoons$ 3-phosphoglycerate +
457 (phosphate)n+1
458
459 In the first and the second reaction, the phosphorylation of the growing PolyP chain
460 requires 1 mol ATP per mol phosphate added. In algae. However, in the second reaction,
461 one may hypothesize that 1 mol 1,3-bisphosphoglycerate could be regenerated from 1
462 mol 3-phosphoglycerate by phosphoglycerate kinase at the expense of 1 mol ATP. The
463 total cost (as ATP) of the second reaction is thus 2 mol ATP per mol phosphate group
464 added to the PolyP chain. Note that throughout this study, we assume that PolyP is
465 synthesized using the first scheme, i.e. the most parsimonious biochemical pathway.
466

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 ort hophosphate 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 [61-63].

Using a Redfield atomic ratio of 106:1 C:P, the total organic cell carbon concentrations in freshwater (23.23 mol C L_{cell}^{-1}) or marine (11 mol C L_{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 kesslerii*), the studies of Hassler and Wilkinson [64] and Wolterbeek et al. [65] 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^{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 [66] and modelling of Cd uptake in the freshwater alga *C. reinhardtii* exposed to a free Cd^{2+} concentration of 7 nM (toxic on

513 cell division, but not yet toxic on cell volume yield) for 60 h suggests that Cd efflux is
514 negligible [67]. Moreover, Angel et al [68] did not observe any Cu efflux by *P. tricornutum*
515 cells previously exposed to 15 μg L^{-1} total dissolved Cu for 72 h (a Cu concentration and
516 exposure time inhibiting cell biomass by more than 70% relative to that of the control).
517
518 **7. Energy cost of As reduction and methylation**
519
520 **7.1 Biochemical pathway and energy cost of As reduction and methylation**
521
522 The biochemical pathway leading to methylation and reduction of As is poorly known in
523 algae. Most of the knowledge we have of this pathway comes from the work performed
524 in vertebrates and fungi (Hughes 2002, Bentley and Chasteen, 2002). Briefly, inorganic
525 arsenate [iAs(V)] is thought to be reduced to inorganic arsenite [iAs(III)] followed by
526 successive oxidative methylation and reduction reactions, which produce sequentially
527 monomethylarsonic acid or MMA(V), monomethylarsonous acid or MMA(III),
528 dimethylarsinic acid or DMA(V), dimethylarsinious acid or DMA(III) and
529 trimethylarsenic oxide or TMAO(V), which is reduced to trimethylarsine gas or
530 TMA(III) (reaction 3). This section describes the biochemical pathway and energy cost
531 of As reduction and methylation.
532
533 $iAs(V) + 2\ e^- \rightarrow iAs(III) + CH_3^+ \rightarrow MMA(V) + 2\ e^- \rightarrow MMA(III) + CH_3^+ \rightarrow$
534 $DMA(V) + 2\ e^- \rightarrow DMA(III) + CH_3^+ \rightarrow TMAO(V) + 2\ e^- \rightarrow TMA(III)$
535 (3)

536 Once in the cell, iAs(V) can be reduced to iAs(III) coupled to the oxidation of two GSH
537 molecules [69]. The regeneration of these 2 GSH via glutathione reductase cost 2 NADPH.
538 Subsequently, iAs(III) could be complexed to GSH and/or exported out of the cells
539 perhaps via silicic acid transporters (Lsi2), acting in silicic acid efflux with higher cytosol
540 than external silicic acid concentration, as demonstrated in rice [70]. However, the gene
541 homolog coding for Lsi2 is not present in eukaryotic algae
542 (http://www.ncbi.nlm.nih.gov). iAs(III) can also be reduced with GSH and can be
543 methylated with As methyltransferases as shown in thermophilic and acidophilic algae [71].
544 Note that this enzyme has been found in several algae species
545 (http://www.ncbi.nlm.nih.gov). The methyl group used by As methyltransferase could be
546 transferred by the S-adenosylmethionine (SAM or AdoMet) cycle [72]. In this cycle, 5-
547 methyltetrahydrofolate (N5-Me-THF) is used as a methyl donor in the SAM cycle and
548 can be regenerated from 5N,10C methylene tetrahydrofolate (5N,10C methylene THF).
549 5N,10C methylene THF can be synthesized via two pathways at the same energy cost: 1)
550 the serine-glycine cycle and 2) synthesis of serine from triose phosphate, conversion of
551 serine to glycine and glycine catabolism [23]. Here we start by computing the cost of
552 5N,10C methylene THF synthesis by both pathways before calculating the cost of the
553 methyl transfer reactions in the SAM cycle.
554
555 The first possibility considered is that the 5N,10C methylene THF used in the synthesis
556 of N5-Me-THF is assumed to come from the serine-glycine cycle. To allow for 5N,10C
557 methylene THF synthesis in the dark requires that the starting point for the cycle is taken
558 as serine derived from glycolytic or photosynthetic triose phosphate in glycolysis. The

559 serine-glycine cycle is initiated by the synthesis of 2 mol triose phosphate in
560 photosynthesis, which requires 6 x 2 or 12 mol NADPH and 9 x 2 or 18 mol ATP.
561 Conversion of 2 mol triose phosphate to 2 mol 3-phosphoglyerate produces 2 mol NADH
562 and 2 mol ATP. 2 mol 3-phosphoglyerate are converted to 2 mol glycerate by a
563 phosphatase and the 2 mol glycerate are converted to 2 mol hydroxypyruvate with
564 production of 2 mol NADH. Conversion of 2 mol hydroxypyruvate to 2 mol serine using
565 external NH_4^+ and the GS-GOGAT pathway requires 4 mol ATP and 2 mol NAD(P)H.
566 Overall, the production of 2 mol serine from 6 mol H_2O, 6 mol CO_2 and 2 external mol
567 NH_4^+ costs 10 mol NAD(P)H and 20 mol ATP. Conversion of 2 mol serine to 2 mol
568 glycine converts 2 mol THF to 2 mol 5N,10C methylene THF. Conversion of 2 mol
569 glycine to 1 mol serine involves production of 1 mol CO_2, 1 mol NH_4^+ and 1 mol NADH.
570 This initial reaction sequence leading to the synthesis of 1 mol serine converts 6 mol
571 CO_2, 6 mol H_2O, 2 external mol NH_4^+ and 2 mol THF to 1 mol serine, 3 mol CO_2, 1 mol
572 internal NH_4^+ and 2 mol 5N,10C methylene THF . The overall cost is 9 mol NAD(P)H
573 and 20 mol ATP with a cost of 40.5 mol absorbed photons to produce 9 mol NAD(P)H
574 and13.5 mol ATP by non-cyclic electron flow, and 6.5 mol absorbed photons to produce
575 the remaining 6.5 mol ATP by cyclic electron flow, i.e. 47 mol absorbed photon to
576 convert 2 mol THF to 2 mol 5N,10C methylene THF, or 23.5 mol photons per 1 mol THF
577 converted to 1 mol 5N,10C methylene THF.

578

579 Subsequent to this, the serine-glycine cycle requires the synthesis of another molecule of
580 serine besides the serine from the initial round of conversion of 2 mol THF to 2 mol
581 5N,10C methylene THF. Synthesis of the additional serine, using internal NH_4^+ from the

582 conversion of 2 glycine to 1 serine in the initial round costs 5 mol NAD(P)H and 9 mol
583 ATP per serine. Conversion of the 2 serine to 2 glycine, and then conversion of the 2
584 glycine to 1 serine, produces 2 mol 5N,10C methylene THF, 1 mol CO_2, 1 mol NADH
585 and 1 mol internal NH_4^+. This gives an overall cost of 4 mol NAD(P)H and 9 mol ATP in
586 producing 2 mol 5N,10C methylene THF, i.e. 2 mol NAD(P)H and 4.5 mol ATP for the
587 production of 1 mol 5N,10C methylene THF. The production of 2 mol NADPH and 3
588 mol ATP costs **9 mol absorbed photons** in non-cyclic electron flow, and production of
589 the remaining 1.5 mol ATP by cyclic electron flow costs **1.5 absorbed photons**, a total
590 of **10.5 mol absorbed photons**.

591

592 5N,10C methylene THF can also be synthesized from CO_2, PAR, NH_4^+ and water via a
593 second pathway involving the production of serine from triose phosphate in glycolysis,
594 the conversion of serine to glycine and the catabolism of glycine to CO_2, NH_4^+ and 2
595 molecules of 5N,10C methylene THF. Synthesis of 1 mol triose P in photosynthesis from
596 H_2O and CO_2 uses 6 mol NADPH and 9 mol ATP. Conversion of 1 mol triose phosphate
597 to 1 mol 3-phosphoglyerate produces 1 mol NADH and 1 mol ATP; phosphatase activity
598 produces 1 mol glycerate from 1 mol 3-phosphoglycerate and this 1 mol glycerate is
599 converted to 1 mol hydroxypyruvate with production of 1 mol NADH. Conversion of 1
600 mol hydroxypyruvate to 1 mol serine using external NH_4^+ and the GS-GOGAT pathway
601 requires 2 mol ATP and 1 mol NAD(P)H. Overall, the production of 1 mol serine from
602 H_2O, CO_2 and external NH_4^+ costs 5 mol NAD(P)H and 10 mol ATP. Conversion of 1
603 mol serine to 1 mol glycine converts 1 mol tetrahydrofolate (THF) to 1 mol 5N,10C
604 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 NH_4^+ produced in glycine catabolism can be used in amination of hydroxypyruvate, thus saving the 1 ATP used in NH_4^+ 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**

We then need to describe the reactions of the SAM cycle (and compute their energy cost) 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. Overall, the SAM cycle,

627 which transfers a methyl group to As methyl transferase, requires 1 NADPH and 1 ATP,
628 but also generates 1 NADH.
629
630 In summary, iAs(V) is first reduced to iAs(III) at a cost of 2 NAD(P)H for the
631 regeneration of two GSH molecules. iAs(III) can then be sequentially reduced by GSH
632 and methylated by the SAM cycle three times each yielding TMAO and TMA. The three
633 reduction steps proceed at a potential cost of 6 NADPH if GSH reduces As(V) species
634 and has to be regenerated. The mechanistic cost of reduction/methylation of 1 mol As(V)
635 (without taking into account the biosynthetic cost of methyl groups) is 11 mol NAD(P)H
636 and 3 mol ATP coupled to the production of 3 mol NADH, i.e. a cost of 8 mol NAD(P)H
637 and 3 mol ATP. Adding the cost of 5,10 methylene THF synthesis for the three rounds of
638 the SAM cycle (required to transfer three methyl groups to oxidised inorganic or organic
639 As(III) species), the total energy cost of iAs(V) reduction and methylation from CO_2,
640 PAR, and water is 14 mol NAD(P)H and 16.5 mol ATP.
641
642 Conversion of the ATP and NAD(P)H cost of As reduction and methylation to photon
643 requirements uses the values of 9 absorbed mol photons to produce 2 mol NADPH and 3
644 mol ATP in non-cyclic electron flow, which is equivalent to 4.5 absorbed mol photons
645 per 1 mol NADPH and 1.5 mol ATP. In cyclic electron flow, 1 absorbed mol photon is
646 required to produce 1 mol ATP [12, 19]. The production of 14 mol NADPH (as well as the
647 21 mol coupled ATP) by non-cyclic electron flow required for the reduction/methylation
648 of 1 mol As(V) costs 63 mol photons (14x4.5). The 4.5 mol ATP in excess would have
649 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 [73]. 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} µmol As per mg DW algae per day and As reduction rate less than 10^{-11} mol As per mg DW algae per day.

673 According to Nalewajko [48], a strain of *Chlorella* (*Chlorella pyrenoidosa*) has 255 µg DW
674 per µL of biovolume (or 255 mg DW per 10^{-3} L of biovolume). Converting the data of
675 Knauer and Hemond [73] on an "approximate" basis of cell biovolume instead of cell dry
676 weight means that the As cell quotas are less than 2.6 x 10^{-5} mol As L_{cell}^{-1} corresponding
677 to an As accumulation rate less than 26 µmol As per L_{cell}^{-1} per day and As reduction rate
678 less than 2.55 µmol As per L_{cell}^{-1} per day.
679
680 In the second study considered here, i.e. the paper of Maeda et al. [74], 67 mM inorganic
681 As(V) started to decrease the cell yield (20% cell yield inhibition) of *C. vulgaris* obtained
682 after growing a culture up to the stationary growth phase at As cell quotas (only rinsed
683 with water!) of around 18-19 mg As per g dry weight or 0.24-0.26 mmol As per g DW or
684 240 to 260 nmol As per mg DW. In this case, the cellular As concentration required to
685 inhibit cell growth appears to be very high (around 58 to 62 mM assuming 255 µg DW
686 per µL of biovolume [48]) although these cell concentrations overestimate the true
687 intracellular As concentration since the As adsorbed onto the cells was not removed in
688 that study. Moreover, they only measured the cell yield in the stationary phase.
689 Measuring As toxicity in the stationary growth phase could underestimate As toxicity if
690 algae deplete dissolved As concentration in solution and then resumed their growth
691 through As biodilution and then may reach higher final cell density in the stationary
692 growth phase (relative to the case where dissolved As concentration is buffered in
693 solution) after a longer log growth phase than the As-free control.
694

695 The third study was that of Karadjova et al. [75]. In that study, the As cellular quotas (rinsed
696 with phosphate) (measured after 72-h of exposure to inorganic As(V) or As(III)) close to
697 the threshold of As toxicity on growth rate of the marine green alga *Chlorella salina* was
698 around 10^{-18} mol As per cell at three different external phosphorus concentration
699 (compared Fig. 2 and Fig. 3). In their Fig. 5A, the relative proportion of each As species
700 (As(V), As(III), MMA, DMA, DHF) accumulated within the algae is shown. Taking a
701 cell volume of *C. salina* of 116.29 μm^3 per cell [76], the intracellular As concencentration
702 required to inhibit *C. salina* growth is around 8.6 µmol As per L_{cell}.
703
704 Fourth, Levy et al. [77] calculated a LOEC for a 72-h exposure to As(III) of 3.75 mg L^{-1} in
705 *Monoraphidium arcuatum*. For As(V), the LOECs were 0.081 and 1.91 mg L^{-1} for initial
706 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
707 respectively). At 125 µg initial As(V) L^{-1} and 0.15 mg P L^{-1} (i.e. an As(V) concentration
708 close to the LOEC 72 h), the mean As cell quota (cells rinsed with phosphate) after 72 h
709 was 1200 x 10^{-18} g $cell^{-1}$ (16 x 10^{-18} mol $cell^{-1}$). At 1000 µg As(V) L^{-1} and 1.5 mg L^{-1}
710 (close to the LOEC of 1.91 mg L^{-1}), mean As cell quota (cells rinsed with phosphate)
711 after 72 h was 400 x 10^{-18} g $cell^{-1}$ (5.3 x 10^{-18} mol $cell^{-1}$). Taking a relatively modest cell
712 volume of 42 μm^3 [78], the intracellular As concentration required to inhibit *M. arcuatum*
713 growth is around 1.2 to 3.7 x 10^{-4} mol L_{cell}^{-1}.
714
715 Fifth, in the study of Pawlik-Skoronska et al. [79], a 24-h exposure of the green microalga
716 *Stichococcus bacillaris* to a slightly growth-inhibitory As(V) concentration (100 µM) at
717 pH 6.8 and 8.2 resulted in As cell content of 4.04 and 1.27 µmol g^{-1} DW, respectively.

718 Taking a value of the dry weight per cell biovolume of *S. bacillaris* of around 231.7 g
719 DW per L of cell volume [48], we calculated that cellular concentrations of 295 to 936
720 µmol As per L_{cell} are slightly toxic to *S. bacillaris*.
721
722 Sixth, Wang et al. [80] have found that at As(V) concentrations slightly toxic to
723 *Scenedesmus obliquus*, less than 10^4 µg As per g DW was accumulated inside the cells
724 (the cells were rinsed with phosphate). For *Scenedesmus obliquus*, the EC50 on growth
725 rate was 12929 µg intracellular As per g DW (or 172.6 µmol As per g DW) in medium
726 with low P (no EC50 available at high P). For *Chlamydomonas reinhardtii*, the EC50 was
727 3298 and 408 µg As per g DW (or 44.0 and 5.45 µmol As per g DW) in the presence of
728 high and low P, respectively (Table 1). Using values determined in *Chlamydomonas*
729 *angulosa* of 317.8 g DW per L of algae and in *Scenedesmus quadricauda* of 498.5 g DW
730 per L of algae, the EC50 on a cell volume basis can be estimated, i.e. an EC50 of 86
731 mmol As per L_{cell} for *S. obliquus* and EC50s of 1.7 to 14 mmol As per L_{cell} for *C.*
732 *reinhardtii*.
733
734 **7.3 Calculating the energy cost of As biotransformation in relation to As**
735 **accumulation**
736
737 It has been shown that up to around 30% of cellular As(V) can be reduced into As(III) [75,
738 81] and part of this As(III) is then methylated although at rates typically much lower than
739 As(V) reduction rate [82] and the methylated As species account for a low proportion of
740 total cellular As [75, 77]. There is also a part of cellular As(III) that is excreted in solution.

741 This As efflux is thought to be relatively slow in *Chlorella salina*. Indeed, this species
742 exposed to 10 µM As(V) for 72 h accumulated around 10^{-17} mol As per cell, but excrete
743 in the dissolved phase 55 to 65% of the total cellular As over 72 h of depuration [75].
744 Assuming a specific growth rate of 1 d^{-1} or a doubling time [Ln(2)/µ] of 0.693 d or 16.6 h
745 and a constant As loss rate over the 72 h exposure, only 13 to 15% of cell As is lost
746 during the period for growing a whole cell. Taking a mean cell volume of *C. salina* of
747 116 μm^3 per cell [76], the As cellular concentration lost during a period of one cell doubling
748 is only around 11 to 13 µM As. Assuming an energy cost of 1 ATP per mol of As
749 exported from the cells, based on the ATP cost for transport of numerous ions across the
750 plasmalemma [60] and considering a cost of 1 mol absorbed photons per mol ATP produced
751 via cyclic electron flow, the energy cost of As(III) efflux is only 2.0 to 2.3 x 10^{-6} % of the
752 total energy cost for growing a *C. salina* cell.
753
754 Considering that the proportion of total As cellular concentration detoxified by reduction
755 and methylation is lower than 30% and that As efflux is slow, then, even with some
756 allowance for the slow As efflux, the steady-state cellular concentration of detoxified As
757 is expected to be lower than the total measured cellular steady-state As concentration. For
758 algal cells accumulating at most around 50 mM As(V) concentrations at the onset of As
759 toxicity on growth (20% cell yield inhibition after several days of growth) [74], the energy
760 cost invested in As detoxification is therefore probably lower than 2.95 mol absorbed
761 photons per L_{cell} (assuming that all cellular As is detoxified and using a cost of 58.5 mol
762 absorbed photons per mol reduced/methylated As(V), see section 7.1), which is 0.5% of

763 the total photon energy cost for growth of a freshwater cell (557.52 mol absorbed photons
764 per L_{cell} ; see method section of the main text body).
765

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 [83-86]. 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 [84, 85]. Since plasmalemma redox enzymes reducing Cu(II) and Fe(III) exist in phytoplankton [87-89] 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 [89]. The Fre1p enzyme couples Fe(III) reduction into Fe(II) to NADPH oxidation [90].

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 unspecifc Hg reduction is **3 mol absorbed photons per mol of reduced Hg(II).**

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

The freshwater alga, *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 [84]. Using a cell volume of 22.3 pL [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} [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, *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} [85]. 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 [93], it means that the Hg reduction rate of *T. weissflogii* is around 67 nmol L_{cell}^{-1} h^{-1}. Thus for a specific growth rate of 1 d^{-1} [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. [83] 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 [94]. These Hg reduction rates when converted on a biovolume basis vary between 1.7 to 234 nmol Hg L_{cell}^{-1} h^{-1}, which is of the same order of magnitude than the Hg reduction rate measured by Morelli et al. [85].

Wu and Wang [86] 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_{cell} determined by Ho et al. [95] in 15 marine phytoplankton species, the above Hg(0) production rate measured by Wu and Wang [86] is equal to 0.66 pmol L_{cell}^{-1} h^{-1}, which is much lower than the Hg reduction rates determined by Mason et al. [83] and Morelli et al. [85].

Therefore, at low nM Hg(II) exposure concentrations associated to the reduction of one micromole Hg per L_{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_{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_2O_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 [97]. 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

879 [98]. Steady-state total GSH concentration is usually tightly regulated in freshwater algae
880 species at a fixed total concentration in response to low non-growth inhibitory Cd and Cu
881 exposure [33, 44, 99, 100]. By contrast, GSH cellular concentration (amount of GSH per L_{cell}) is
882 expected to decrease at higher toxic Cd concentrations on growth even though GSH cell
883 quotas did not change significantly [33, 44] since algal cell volume sensitively increase at
884 [Cd] that inhibits the growth of *C. reinhardtii* [101]. Note that at high growth-inhibitory Cu
885 concentrations, GSH concentrations decreased in *C. vulgaris* [100]. Also, exposure to the
886 metalloids As (concentration inhibiting by 12 to 27% the growth rate) and Sb (non-
887 inhibitory concentration) respectively caused an increase and a decrease in GSH cell
888 quotas of *Scenedesmus vacuolatus*, but potential changes in cell volume were not
889 reported and calculation of GSH cell concentration cannot be performed [41]. Once GSH is
890 converted in GSSG in the presence of ROS, GSH can be rapidly regenerated via
891 glutathione reductase at a cost of only 1 NAD(P)H [102]. Taking a second order rate
892 constant of GSH oxidation by the •OH radicals [103] of 1.4×10^{10} M^{-1} s^{-1} and assuming 10^{-18}
893 M •OH in unstressed cells [104], it follows that over the period of a cell doubling (17 h
894 assuming a specific growth rate of 1 d^{-1}), less than 0.1% of total cellular GSH is oxidized
895 by •OH in unstressed cell. Regeneration of 0.1% of a cellular pool of 10 mM GSH costs
896 only 3×10^{-5} mol absorbed photons, i.e., less than around 10^{-5} % of the total energy cost
897 for growth. Consequently, the energy cost of GSH regeneration (only considering GSH
898 oxidation by the radical •OH), particularly in algal cells exposed to metal(loid)
899 concentrations close to the NOEC, is likely a low proportion of the total energy cost for
900 growth.
901

902 Ascorbate is another major antioxidant in algal cells present at millimolar concentrations.
903 Ascorbate concentrations in a range of fresh and marine phytoplankton species, which
904 were compiled in 1995 by Raven [105], were approximately between 0.4 and 28 mmol L_{cell}^{-1}
905 [1]. Subsequent measurements by Urzica et al. [106] of the mean intracellular ascorbate
906 concentration in *C. reinhardtii* cells cultivated under optimal growth conditions yielded
907 values around 0.1 mmol L_{cell}^{-1} [106] using a critical cell volume of 140 fL at which
908 synchronized *C. reinahrdtii* cells (12 h ligh: 12h dark cycle) divide [107]. Also, Sunda et al.
909 [103] reported intracellular ascorbate concentrations of around 6 mM in *E. huxleyi*. Oxidized
910 ascorbate can be reduced back into ascorbate by MDA reductase (MDAR) at the cost of
911 only 1 NAD(P)H or 3 mol absorbed photons (i.e., 4.5 mol photons per mol NAD(P)H by
912 non-cyclic electron flow minus the cost saved for synthesizing the coupled 1.5 mol ATP
913 or 1.5 mol photons by cyclic electron flow). Taking a second order rate constant of ASC
914 oxidation by the •OH radicals of 1.1×10^{10} M^{-1} s^{-1} tabulated in Sunda et al. [103] and
915 assuming 10^{-18} M •OH in unstressed cells [104], it follows that over the period of a cell
916 doubling (17 h assuming a specific growth rate of 1 d^{-1}), less than 0.1% of total cellular
917 ASC is oxidized by •OH in unstressed cell. Regeneration of 0.1% of a cellular pool of 6
918 mM costs only 1.8×10^{-5} mol absorbed photons, i.e., less than 10^{-5} % of the total energy
919 cost for growth. Consequently, the replacement cost of ASC (only considering ASC
920 oxidation by •OH), particularly in algal cells exposed to metal(loid) concentrations close
921 to the NOEC, is likely a low proportion of the total energy cost for growth.
922
923 The following reasoning further illustrates that regeneration of reduced ASC and GSH is
924 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 regenerated 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 [108]. 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.

947 The basal intracellular concentration of free proline in freshwater phytoplankton is in the
948 range 100 μM to 3 mM [109-112], based on published measurements of free proline quotas in
949 algae and conversion with cell volumes measured in each species [113]. By comparison,
950 internal proline concentrations in marine phytoplankton vary between 2 mM and 340 mM
951 at the salinity of seawater (around 600 mM NaCl) [114, 115]. Intracellular proline
952 concentrations in freshwater phytoplankton were shown to increase in response to metal
953 exposure. The intracellular proline concentration in *Chlorella vulgaris* was shown to
954 increase from 0.8 to around 8 mmol L_{cell}^{-1} after 10 h of exposure to 2.5 μM total Cu and 5
955 μM total Cr. These Cu and Cr treatments inhibited the algal growth rate by around 15%
956 and 20% respectively. However, the cellular proline level returns to near basal levels
957 after 25 h of exposure [110]. The study of Mallick [116] also showed that the cellular proline
958 concentration in *Chlorella vulgaris* did not significantly increase for a 72-h exposure at
959 low (non-growth-inhibitory) Cu concentrations (72 h), but at toxic Cu concentrations on
960 growth, proline started to increase. In the species *Chlorella* sp., the intracellular free
961 proline concentrations also increased in response to exposures to toxic Cu or Cd
962 concentrations on growth, but the intracellular proline concentrations remained below
963 around 290 μmol L_{cell}^{-1} [109]. The observed transitory induction of proline biosynthesis at
964 internal concentrations below 8 mM is expected to contribute a minor fraction (< 0.1%)
965 of the total energy cost for growth.
966
967 DMSP and GBT are effective antioxidant in marine phytoplankton although less effective
968 scavenger of the •OH radical than ASC and GSH [103, 117, 118]. DMSP and GBT can reach
969 high concentrations usually on the order of 1-200 mM in marine diatoms and

970 coccolithophores [119, 120] although very high DMSP concentration up to around 1 M has
971 been reported in free-living marine dinoflagellates [121]. Since there are no known
972 pathways that can effectively recycle DMSP and GBT (analogous to the recycling of
973 GSSG in GSH by the GR), both antioxidants need to be *de novo* synthesized at relatively
974 high photon energy costs compare to those of other antioxidants. Indeed, *de novo* steady-
975 state biosynthesis of 1 mol DMSP in phytoplankton costs 93 mol absorbed photons
976 whereas *de novo* biosynthesis of 1 mol GBT costs 91 to 108 mol absorbed photons using
977 NH_4+ or NO_3^- as the N source [122]. Although DMSP and GBT could be a costly process
978 for ROS scavenging if their synthesis were strongly up-regulated, no studies, to our
979 knowledge, have shown an increase in DMSP or GBT concentrations in marine
980 phytoplankton exposed to toxic concentrations of metal(loid)s. Indeed, DMSP
981 intracellular concentrations are not up-regulated (and not totally regenerated) in the
982 marine algae *E. huxleyi* exposed to a growth-inhibitory Cu concentration (1 nM Cu^{2+}),
983 but rather are decreased under Cu stress [103].
984
985 Even though turnover rates of DMSP in metal(loid)-exposed cells are currently unknown,
986 we attempted to constrain the probable energy cost due to DMSP regeneration by *de novo*
987 DMSP biosynthesis. According to the following reasoning, significant depletion of
988 intracellular DMSP due to oxidation by ROS (and significant energy loss due to DMSP
989 oxidation) is unlikely at least at metal(loid) concentration close to the NOEC. Indeed,
990 based on the published rate constant of DMSP oxidation by •OH ($k = 9 \times 10^8$ M^{-1} s^{-1}) and
991 $\bullet O_2^-$ ($k = 8.3$ M^{-1} s^{-1}) radicals [123], an intracellular •OH concentration in unstressed cells of
992 10^{-18} M [104] and a chloroplastic $\bullet O_2^-$ concentration of around 10^{-9} M [124], less than 10^{-4} % of

993 intracellular DMSP should be oxidized by these two radicals over a period of one day.
994 This suggests that even if a considerable increase in ROS production under a severe
995 metal(loid) stress occurred, the experimentally measured cellular DMSP pool would not
996 decrease significantly. Even at the high intracellular DMSP concentrations measured in
997 marine dinoflagellates (≈1 M) and assuming a doubling time of 17 h ($\mu = 1\ d^{-1}$), less than
998 10^{-6} M DMSP is expected to be consumed by reaction with •OH and $\bullet O_2^-$. Assuming that
999 the total cellular pool of DMSP is kept constant due to *in vivo* DMSP production in
1000 control cells, then the photon cost of DMSP regeneration is 93×10^{-6} mol absorbed
1001 photons, i.e., less than 10^{-2} % of the total photon energy cost for growth. Hence, even for
1002 a one or two orders of magnitude increase in •OH and $\bullet O_2^-$ concentrations in cells
1003 exposed to metal(loid)s, regeneration of DMSP by *de novo* DMSP synthesis would likely
1004 be a minor energy expenditure. Likewise, since GBT is a less effective hydroxyl radical
1005 scavenger than DMSP [118], the cost for maintaining the intracellular pool of GBT constant
1006 by *de novo* GBT synthesis in conditions of oxidative stress is also expected to be minor.
1007
1008
1009 **11. Lipophilic non-enzymatic antioxidants**
1010

1011 Intracellular concentrations of lipophilic carotenoids that specifically protect the
1012 chloroplast apparatus from ROS generally remain constant, or decrease, in microalgae
1013 exposed to Cd or Cu. Only in a few cases did carotenoids increase, but at growth-
1014 inhibitory metal concentrations, suggesting that they are not on the first line of defense
1015 against ROS in algae. Indeed, exposure of *Scenedesmus* sp. to a toxic Cu concentration

(on O_2 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 *Nitszchia 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 ß-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(loid)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.

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